Chronic Degenerative Radiculomyelopathy: A study of the pathology and pathogenesis

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Abstract

Chronic degenerative radiculomyelopathy (CDRM) is a well-recognised neurodegenerative disease of unknown aetiology which affected large breed dogs, primarily the German shepherd dog (GSD). Previous studies had identified the major clinical signs of pelvic limb ataxia and weakness resulting from degeneration of tracts in spinal cord white matter. This clinical entity has been recognised since 1973, most reports concentrated on the clinical aspects of the disease and the pathology of the spinal cord. This project is the first to study a large number of affected dogs repeatedly throughout the course of disease. The previously reported clinical signs have been confirmed and the relationship between age of onset and rate of deterioration has been addressed for which no definite correlation was found.

Clinical and pathological similarities between CDRM and neurodegenerative disorders due to vitamin E deficiency in horses and humans had implicated vitamin E as a potential factor in the aetiology of CDRM. This stimulated a study of serum vitamin E concentrations. Data presented in this thesis suggests that affected GSDs do not have significantly lower serum vitamin E concentrations than other breeds of dog. In contrast, GSDs with CDRM appear to have elevated levels of serum vitamin E in comparison with the general canine population.

The pathology of the spinal cord is consistent with previously-described spinal cord pathology. However detailed examination of the brains of affected dogs revealed novel pathological changes in specific brain nuclei. Such changes included neurones with eccentric nuclei, chromatolytic neurones and neuronal loss often with an associated gliosis. These changes affected the red nucleus, lateral vestibular nucleus and lateral (dentate) nucleus to varying extents. Such changes were found consistently in CDRM dogs but only rarely in dogs with focal spinal cord lesions. Furthermore, gliosis in the red nucleus was found only in the dogs with CDRM.

Due to the unusually high incidence of CDRM in one breed of dog and the discovery of at least two pairs of affected littermates, the investigation of a possible genetic factor was indicated. Following a literature search for diseases in other species with clinical and pathological similarities to CDRM, a working hypothesis was established: CDRM is caused by a CAG trinucleotide repeat expansion in an unknown gene. A number of molecular biological techniques were employed to test this theory, including the Repeat Expansion Detection (RED) technique. This work is still in progress but there is some evidence, still inconclusive, that CDRM may be the result of a trinucleotide repeat expansion.

To my parents

and

In memory of Douglas Hodges, whose generosity allowed this work to be carried out.

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Declaration

I, Pamela Elizabeth Jean Johnston, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for the award of a degree at any other University.

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1. Introduction

1.1 Background Information

1.1.1 Establishment of the nomenclature

The occurrence of a progressive pelvic limb ataxia and paresis in the older German shepherd dog (GSD) had been recognised for many years. Originally the clinical signs were attributed to the presence of ossified plaques in the dura mater which commonly developed at the cervical and lumbar enlargements, usually on the ventral and ventrolateral aspects of the spinal cord. In extreme cases a bony tube formed around the cord (Morgan, 1968). This ossification, which was relatively common in older dogs, was called chronic ossifying pachymeningitis or spinal dural ossification.

These plaques could not have accounted for the clinical signs seen in chronic degenerative radiculomyelopathy (CDRM). Morgan (1968) showed that dural ossification was a relatively common finding in middle to old age dogs of large breeds but occurrence did not necessarily coincide with signs of pelvic limb ataxia or weakness. In his study, approximately two thirds of all dogs over two years of age had evidence of dural ossification. Of the 52 cases which showed dural ossification only 12 had clinical signs of abnormal gait or hindquarter weakness; a further three dogs had clinical signs but no evidence of dural ossification. The position of the majority of plaques at the cervical and lumbar enlargements would have resulted in a lower motor neurone lesion (LMN) whereas CDRM involved an upper motor neurone lesion (UMN) (Griffiths and Duncan, 1975a). In addition, the occurrence of plaques at the cervical enlargement should have caused concurrent thoracic limb and pelvic limb dysfunction, which did not occur.

The condition was first described as degenerative myelopathy by Averill (1973). When they found degenerating fibres in the lumbar dorsal nerve roots of a number of affected dogs, Griffiths and Duncan (1975a) proposed the name chronic degenerative radiculomyelopathy (CDRM). German shepherd dog myelopathy was another term used to describe the condition. In this account the disease will be referred to as CDRM.

1.1.2 Occurrence

This disease had been described in a number of large breeds and large breed crosses such as German shepherd dog (GSD), Irish setter, Collie cross (Averill, 1973), Rough collie, Rhodesian ridgeback and Labrador cross (Griffiths and Duncan, 1975a). Most cases reported had been in the GSD. Of the studies completed to date, the percentage of affected dogs which were GSD ranged from 56% (9/16)

(Griffiths and Duncan, 1975a) to 82% (18/22) (Averill, 1973). These relatively small studies did not give an accurate picture of the actual occurrence within breeds and precise information regarding incidence was difficult to obtain. Experts in the field (Griffiths, personal communication, 1995) suggested that about 90% of affected dogs are GSDs. In addition, anecdotal evidence (GSD breeders, personal communication, 1996) suggested that up to 20% of the total GSD population may develop clinical signs suggestive of CDRM at some stage in their lives.

1.1.3 Aetiology

Prior to 1973 the clinical signs were ascribed to the presence of dural bone plaques or multiple small intervertebral disc protrusions (Morgan, 1968). Averill (1973) mentioned the similarity with vitamin B_{12} deficiency in man, noting that in man the disease initially involved only the thoracic cord, affected all funiculi and was slowly progressive unless appropriately treated. There was however a fundamental difference between humans and dogs in that the human disease initially exhibited a perivenular pattern and was usually multifocal, whereas CDRM in the dog was topographically continuous. Levels of methylmalonic acid in affected dogs showed no abnormalities, which suggested that normal vitamin B_{12} metabolism was occurring. Williams *et al.* (, 1984) also investigated the possible involvement of vitamin B_{12}. They found that all six affected dogs tested had bacterial overgrowth in their duodenal juice. This abnormality was not associated with any histological changes in jejunal biopsies. These dogs, however, had marked biochemical abnormalities which included the following changes to brush border enzymes: leucyl-2-naphthylamidase was decreased, γ-glutamyl transferase was increased. Marked increases in activities of an endoplasmic reticular enzyme, tris-resistant α-glucosidase and the lysosomal enzymes N-acetyl-β-glucosaminidase and acid phosphatase were also seen. The significance of these changes must be questioned as this study did not include a control population of unaffected dogs. Vitamin B_{12} levels were subnormal in only three of the six dogs.

The above findings suggested that CDRM may occur due to the abnormal absorption of some other nutrient(s) or that the biochemical abnormalities occurred secondary to the neurologic dysfunction. A consideration of nutritional factors which may be involved in CDRM must include vitamin E. Williams *et al.* (, 1985) investigated the serum tocopherol concentrations in a group of seven GSDs with CDRM and concluded that serum tocopherol concentrations were slightly lower in affected dogs (0.5-8.6mg/l) compared to healthy controls (4.4-29.4mg/l). These authors suggested that the enteropathy they saw, and the associated reduction in serum tocopherol levels, may have arisen secondary to CDRM. Alternatively, the

enteropathy may have been the primary lesion responsible for malabsorption of tocopherol and subsequent development of CDRM. It had been shown that deficiency of vitamin E caused neuropathology in other species; for example, degenerative myelopathy/myeloencephalopathy in the horse and ataxia with vitamin E deficiency (AVED) in man. AVED patients were known to respond to the administration of high doses of vitamin E (DiDonato, 1995). In addition, loss of axons and myelin sheaths, especially in the dorsal columns (Pentschew and Schwarz, 1962; Nelson *et al.*, 1981; Southam *et al.*, 1991), had been associated with chronic vitamin E deficiency in rats and rhesus monkeys. As vitamin E was known to work as a free radical scavenger, therefore protecting neuronal cell membranes from peroxidation, its absence could lead to increased membrane fragility and ultimately neuronal cell death.

Several familial neuronal degenerations such as Freidriech`s ataxia, hereditary spastic ataxia and hereditary spastic paraplegia existed in man. These were the result of neuronal atrophy or abiotrophy in which the cell body and proximal nerve fibre remained intact whilst the distal axon degenerated. Although the actual mechanisms were poorly understood, Averill mentioned a possible similarity to CDRM in 1973, suggesting that this process should be considered in CDRM. If correct, he suggested, neuronal loss or atrophy in several important locations such as the spinal ganglia (SpG), red nucleus, rostral colliculus and vestibular nucleus should be present (Averill, 1973).

Griffiths and Duncan (1975a) considered the neuropathology indicative of a "dying back", or distal axonopathy, phenomenon such as occurred in various human neurodegenerative conditions and experimental neurological diseases. Central "dying back" processes had been implicated in conditions such as amyotrophic lateral sclerosis and some of the spinocerebellar disorders (Griffiths and Duncan, 1975a). Griffiths and Duncan considered that no proof of a hereditary factor had been put forward but explained the greater occurrence in large breed dogs to be the result of differential susceptibility due to the longer nerve fibres present compared with small dogs. This did not, however, explain the particular breed predilection seen in CDRM. In studies on equine laryngeal hemiplegia Duncan *et al.* (1974) showed a positive correlation between nerve fibre length and susceptibility to degeneration. Braund (1978) argued that the distribution of lesions in CDRM did not fit the expected pattern of a "dying back" disease. The severity of lesions in the thoracic segments which suggested selective vulnerability of thoracic cord (Averill, 1973; Braund and Vandevelde, 1978) in CDRM remained unexplained despite having been found in viral, nutritional and idiopathic disorders in other species.

Waxman *et al.* (1980b) considered the pathology of CDRM to be indicative of a chronic demyelinating disease rather than an acute inflammatory response. As the immune system was known to play an important role in chronic demyelinating diseases in other species such as multiple sclerosis (MS) in man and experimental allergic encephalomyelitis (EAE) in rats (Bernard and Kerlero de Rosbo, 1992; Hartung and Rieckmann, 1997) the authors investigated the response to thymus-dependent mitogens in seven normal and six CDRM affected dogs. An abnormal T cell function was found in the peripheral blood cells of affected dogs, leucocytes displayed an impaired response to the T cell mitogens concanavalin A (Con A) and phytohaemagglutinin P (PHA). Serum or plasma obtained from dogs with CDRM was not suppressive for normal cells and neither was normal proliferative function restored when peripheral blood cells from affected dogs were cultured in normal serum or plasma, which suggested that any abnormal function of T cells was not due to the presence of serum inhibitory factors. Whether or not this was related to the disease process of CDRM was not known. The results of this study did not explain why depressed lymphocyte responsiveness occurred in dogs with CDRM, nor did it explain why the immunological deficit was seen only in the peripheral blood leucocyte compartment. It was suggested that an autoimmune lymphocyte population may have been activated during the course of CDRM.

In a follow-up study Waxman *et al.* (1980a) showed that CDRM dogs had circulating suppressor cells associated with the impaired peripheral blood response. These findings had not been linked directly to the disease process but they could have indicated either an immunodeficiency problem where depressed T cell function allowed an infectious agent to enter the central nervous system (CNS) or where the suppressor cell population was activated secondarily by the host to regulate an autoimmune event. Barclay and Haines (1994a) studied a small group of dogs (five affected and one unaffected) and suggested that an immune-mediated spinal cord destruction may be occurring, because they found immunohistochemical evidence of immunoglobulin and a component of complement in the spinal cords of affected dogs which was not present in the normal dog. Clemmons (1992) suggested that, despite the lack of direct evidence for CDRM being caused by an infectious agent, it was possible that the disease was the result of a combination of genetic predisposition and exposure to an infectious agent. He reported in 1992 that workers were investigating whether a canine retrovirus infection or "scrapie-like" prion organism was involved, however, neither of these possibilities had been proven (no references were cited on the Clemmons paper).

Undoubtedly, CDRM has a complex aetiology which probably involves several different factors but most authors agreed there was almost certainly a genetic factor due to the very high incidence of the disease in one breed.

1.1.4 Age of Onset

All accounts to date conceded that there was great variation in age of onset, the youngest reported case was five years old, the eldest 14 years while the majority were about nine years old at first presentation.

1.1.5 Clinical Signs

Clinical signs of CDRM had been well documented (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978; Clemmons, 1989; Clemmons, 1992). The dogs showed a slowly progressive pelvic limb ataxia and paresis with loss of proprioception. Initially they scuffed the middle two toes of one or both hind feet. Subsequently they developed problems with circling and with stairs, especially going down, they often scuffed, misjudged distances and showed hypermetria (ataxia in which intended movements overreach the intended goal). The dogs were often affected asymmetrically although both pelvic limbs were usually involved. Disuse muscle atrophy developed over the trunk and hindquarters several months after disease onset. With time, prolonged scuffing resulted in excoriation and ulceration of the feet. Eventually the disease resulted in marked paraparesis, the dogs could not rise without assistance and would pull themselves along with their thoracic limbs. A degree of faecal and urinary incontinence would often develop late in the disease. Dogs maintained beyond this stage could show thoracic limb signs. It had been reported that brain stem involvement eventually occurred (Clemmons, 1992) which could result in a number of signs including asymmetrical tetraparesis, cranial nerve abnormalities and altered mental status. The clinical course was very variable ranging over six to 12 months (Clemmons, 1992) from onset of the disease to complete pelvic limb paraparesis. The clinical signs were inexorably progressive and whilst they may have stabilised for short periods of time improvement did not occur.

On neurological examination conscious proprioception as evidenced by paw position sense, reflex stepping and sway test was severely affected either uni- or bilaterally which depended, to some extent, on the duration of disease. Pedal reflex was usually unaffected while the patellar reflex varied in response to the disease. Hyperreflexia of the patellar reflex, with or without clonus, had been reported (Averill, 1973; Waxman *et al.*, 1980b; Clemmons, 1992). Hyporeflexia and loss of

the patellar reflex had also been reported (Griffiths and Duncan, 1975a). The panniculus reflex remained intact, as did the perineal reflex, until late in the disease.

1.1.6 Diagnosis

It was not possible to diagnose CDRM definitively in life. A presumptive diagnosis could be made based on typical history and clinical signs. Other differential diagnoses such as disc disease, lumbosacral disease or neoplasia could be ruled out by further investigations which included haematology, serum chemistry profiles, urinalysis, CSF analysis, spinal radiographs and myelography. Some authors had reported an unspecified increase in CSF protein and abnormality in electrophoretic pattern (Clemmons, 1992; Clemmons *et al.*, 1995) in dogs with CDRM. Further diagnostic procedures such as electrophysiology, epidurography, discography, computed tomography (CT) and magnetic resonance imaging (MRI) were indicated in specific cases. The results from all these tests were negative in CDRM as definitive diagnosis could be achieved only by histological examination of the spinal cord.

Clemmons (1992) suggested, on the basis that he considered dogs with CDRM to have three to 10 times more circulating immune complexes than normal dogs, that it may be possible to develop serum markers for the specific diagnosis of CDRM. From preliminary work, he suggested the presence of an 85kDa antigen in dogs with CDRM but not in those with other neurological disorders. However, to date, no other authors have mentioned such a possibility.

1.1.7 Treatment

The emphasis in CDRM research over the last 25 years had been to map accurately the neuropathology and investigate the possible aetiologies involved. It had been considered an untreatable disease (Braund, 1987). Clemmons (1989) was the first author to suggest a therapy which may slow down the rate of deterioration in affected dogs. Glucocorticoids, used for the treatment of inflammatory processes or to suppress supposed immune responses, had been used by veterinary surgeons but without any success. Clemmons suggested the use of other immunosuppressive drugs, initially he tried cyclophosphamide and azothiaprine without success. Immunostimulants such as levamisole were also tried but appeared to speed up the rate of progression of the disease. The use of intravenous dimethylsulphoxide (DMSO), which has a number of unproven possible activities including the scavenging of free radicals, and intramuscular cobra venom, had also been tried unsuccessfully.

High doses of vitamin E (2000IU/day), high potency B vitamin complex and epsilon aminocaproic acid (EACA) had all been used as treatments (Clemmons, 1989; Clemmons, 1992) although their efficacy appeared questionable. EACA has anti-protease activity, Clemmons considered that it would therefore be helpful in CDRM as it would block the final step in the inflammatory pathway thus helping to prevent tissue destruction. All authors agreed that maintenance of regular exercise and optimal body weight seemed beneficial to affected dogs.

There was no further evidence which suggested that any of the therapies suggested by Clemmons were beneficial in the treatment of CDRM which was still considered untreatable.

1.1.8 Pathology

The pathology was initially described by Averill (1973) and subsequently by others, all of whom agreed that the lesion involved mainly the spinal cord white matter and affected both the axon and myelin sheath. As previously discussed, before 1973 it was thought that the clinical signs seen in CDRM were attributable to the occurrence of spinal dural ossification. Averill reported irregular oval plaques formed from thin cancellous bone which contained normal bone marrow on the ventral aspect of the dura mater. These did not impinge on the intervertebral space and from their position could not have resulted in the upper motor neurone (UMN) lesions seen in CDRM. Averill also found varying amounts of spondylosis, these varied from small bony spurs on the vertebral bodies to large, hard, wedge-shaped structures which extended underneath the intervertebral space. The incidence of spondylosis was not significantly different between the affected and control groups of dogs and in addition the sites did not correspond with the spinal cord pathology. The spinal cord changes were consistent for all affected dogs. On gross examination of the cord the leptomeninges were slightly thickened, histologically they exhibited slight fibrosis. The white matter ascending and descending tracts were affected to varying degrees throughout the cervical, thoracic and lumbar segments (mid to caudal thoracic segments were the most severely affected). Only the fasciculus proprius appeared to be unaffected. Averill considered these areas to consist of loss of axons and myelin sheaths with an associated astrocytic hypertrophy and hyperplasia. The only grey matter changes noted were a possible loss of ventral horn neurones and an occasional mild perivascular fibrosis of the arterioles in this region. He noted that none of the degenerative changes appeared to extend into either the brain or the peripheral nerves. There were no consistent visceral lesions present in affected dogs.

The pathology was described in more detail by Griffiths and Duncan (1975a). Again they described the presence of degenerative lesions in the spinal cords of all dogs examined but with variations in distribution and severity, these lesions were considered to be typical of Wallerian-type degeneration and affected both the axon and myelin sheath. They noted that any observed clinical asymmetry correlated with the pathological asymmetry. Loss of axons and myelin sheaths was seen in the dorsal funiculi, specifically in the fasciculus gracilis, of cervical and cranial thoracic segments. Occasional axon and myelin sheath loss was also noted in the dorsal funiculi of lumbar and sacral cord segments. In the lateral funiculus the most consistently affected area was the corticospinal tract which was most severely affected in the caudal thoracic segments. Any other fibre loss was scattered and not severe enough to constitute an actual tract involvement. The only grey matter change reported was the presence of increased numbers of astrocytes in the lateral and dorsal horns of grey matter in the lumbar and caudal thoracic segments. Some affected dogs had an occasional SpG neurone which exhibited central chromatolysis. No changes were seen in peripheral nerves or muscles of affected dogs. There was no indication in this paper that the brains had been examined. As the present study identified significant brain pathology this illustrated the necessity for carrying out a complete neuropathological examination.

The report by Braund and Vandevelde (1978) confirmed most of the previous findings, however these authors did not observe any pathological changes in the SpG. They concluded that this may have been due to the clinical and pathological variations in the dogs studied. In addition to previous findings they reported marked axon and myelin loss in the white matter which surrounded the ventromedian fissure. Again they noted that the thoracic cord was the most severely affected region, although both cervical and lumbar segments were also involved.

Clemmons (1989) reported widespread demyelination and axon loss in affected areas of the spinal cord white matter with an associated increase in astrocyte numbers and increase in density of small vascular elements. In the thoracic cord he saw vacuolation in all funiculi, with swollen axons and eosinophilic globules, which represented degenerate and regenerate axons. A number of the vacuoles seen contained macrophages and astrocytes. Clemmons reported finding similar lesions, very occasionally, throughout the white matter areas of the brain (1989).

1.2 Other degenerative myelopathies of dogs

A number of diseases which had clinical and pathological similarities with CDRM had been recognised in other dog breeds. They were all characterised clinically by a

progressive pelvic limb ataxia and paresis. On post mortem examination the spinal cord white matter exhibited degeneration of both axons and myelin sheaths, often with an associated gliosis. The age of onset amongst this group of diseases was, however, very variable.

In 1973 an ataxia with an autosomal recessive mode of inheritance was reported in Jack Russell terriers (Hartley and Palmer, 1973) in Britain and in Sweden. A progressive pelvic limb incoordination and dysmetria with over-protraction of the thoracic limbs commenced at three to six months of age. Affected dogs showed a characteristic "dancing" gait. On pathological examination the two populations varied, although both had focal symmetrical loss of myelinated fibres in the peripheral dorsolateral white matter. In the more severely affected English cases a widespread Wallerian-type degeneration was noted throughout the CNS with degenerative changes in the central auditory pathway and peripheral nerves. It had been suggested that the differences may be due to the different time spans of disease.

A number of authors have reported a condition in a variety of hounds which had been variously named hound ataxia (Palmer and Medd, 1981; Palmer *et al.*, 1984; Palmer and Medd, 1988) or spinal myelinopathy (Sheahan *et al.*, 1991). This condition occurred in the adult animal, two to seven years old, with affected animals exhibiting pelvic limb weakness and incoordination. Most cases had a restricted panniculus localising to mid-thoracic to cranial lumbar segments. Conscious pain sensation remained intact. On pathological examination of the CNS vacuolar degeneration was present in lateral and ventral funiculi throughout the length of the spinal cord. Astrocytic proliferation and myelin debris were common. Changes in the spinal cord grey matter were uncommon, but chromatolysis was observed in the lateral vestibular nucleus and in thoracic neurones in segments T7 and T8 in two cases out of sixteen (Palmer *et al.*, 1984). In all other cases the pathology observed in the brain stem was indicative of tract degeneration. The changes suggested a primary myelinopathy, unlike CDRM which appeared to have simultaneous degeneration of both the axon and myelin sheath. The occurrence of this disorder amongst foxhounds coincided with a change in diet to include a high proportion of ruminant stomachs (tripe). Subsequent alteration to include a much higher proportion of meat had resulted in decreased incidence of the disease. Thus, a clear association had been established between the occurrence of hound ataxia and the feeding of ruminant stomachs which possibly resulted in decreased methionine levels and increased methionine synthetase activity in serum. It had been previously suggested that neurological deficits in subacute combined degeneration in man could have resulted from a methyl group deficiency associated with decreased levels

of methionine and a concomitant decrease in levels of S-adenosylmethionine. The suggestions for the aetiology of hound ataxia have never been confirmed, although the incidence had reduced dramatically since the diet was altered to include more meat.

Bichsel and Vandevelde (1983) described three related Siberian huskies with a late-onset progressive pelvic limb ataxia. In the two cases which could be examined muscle tone and flexor reflexes in the pelvic limbs were weak, whereas patellar reflexes were exaggerated. Pain sensation remained intact but proprioceptive positioning of the pelvic limbs was slow. These dogs all developed urinary incontinence as the disease progressed. At post mortem all three dogs had similar changes which comprised of disseminated vacuolation of the white matter, sometimes associated with axonal swelling and necrosis. These changes were found at all levels of the cord, especially in the ventral and lateral columns. Minimal gliosis was observed and there was no evidence of inflammation. The thoracic segments were the most severely affected, particularly in the peripheral white matter. The changes were asymmetrical and were not attributable to specific tracts. The affected dogs were closely related, two littermates and their mother, which suggested a possible hereditary basis to the disease.

A progressive pelvic limb ataxia had been reported in a five month old Pyrenean mountain dog (Wright and Brownlie, 1985). Diffuse axonal degeneration was noted in the dorsolateral, lateral and ventral white matter extending from the medulla to the fifth lumbar segment. The most severely affected segments were C8 to L2. The degenerative changes involved both the axon and myelin sheath. The SpG and the spinal cord grey matter were unaffected. Changes found in the brain included axonal swelling and degeneration in the spinocerebellar tracts, caudal and middle cerebellar peduncles and the cerebellar folia. No attempt had been made to determine the underlying cause of this disease.

In 1995 a degenerative myelopathy was reported in a ten year old Miniature poodle (Matthews and de Lahunta, 1985). A progressive pelvic limb ataxia and paresis developed with loss of proprioception, which initially affected only one pelvic limb. As the disease progressed hypertonia and hyperreflexia became evident in both pelvic limbs. Post mortem examination of the spinal cord revealed extensive diffuse degeneration of the white matter in all funiculi at all levels, which involved both axons and myelin. Again, the thoracic cord was the most severely affected. The spinal nerve roots were unaffected in this case. No cause was established.

A progressive neurological disorder had been reported in a young adult Ibizan hound (Summers *et al.*, 1995). The ataxia was obvious in the pelvic limbs from the

time of ambulation and progressed to involve the thoracic limbs. The gait was spastic and dysmetric with overflexion and abduction which led to a distinctive bobbing, bouncing type of gait. Patellar reflexes were absent. On microscopic examination of the spinal cord there was symmetrical degeneration of ascending and descending tracts in all funiculi and at all levels of the spinal cord although the thoracic cord was most severely affected. Unlike CDRM, the pathology extended into specific peripheral nerves where there were dilated myelin sheaths which contained intact axons. This condition essentially represented a diffuse nervous system degeneration with myelopathy and neuropathy which mainly affected axons. Pedigree analysis supported an autosomal recessive pattern of inheritance for this condition.

A degenerative myelopathy with some resemblance to CDRM had recently been reported in the young GSD (Longhofer *et al.*, 1990). The two affected animals were six and seven months of age. They exhibited a progressive pelvic limb ataxia and weakness. These dogs had very poor muscling of their hindquarters and normal conscious proprioception which distinguished them from dogs with CDRM. Pedal and patellar reflexes were intact but one dog had clonus of the patellar reflex. The pathological findings were similar in both dogs and involved symmetrical diffuse degeneration of axons and myelin with mild gliosis. These changes occurred predominantly in the lateral and ventral funiculi. The thoracic cord was most severely affected. Axonal degeneration was a prominent feature in this disease with many axonal spheroids and macrophages in affected areas of white matter. The only changes seen in the brain involved occasional degenerating axons in cerebellar white matter. The SpG and spinal nerve roots were all normal. Due to the significant differences between this condition and CDRM it was suggested that the pathogenesis may be unrelated. In addition, since the dogs were both thin and poorly muscled, a possible hereditary metabolic disorder was postulated.

There were other progressive neurodegenerative conditions which had been reported in a number of different dog breeds. These conditions were too dissimilar to CDRM to warrant discussion in this thesis. Examples of these were hereditary myelopathy in the Afghan hound (Cockrell *et al.*, 1973; Cummings and de Lahunta, 1978), leukoencephalomyelopathy in the Rottweiler (Gamble and Chrisman, 1984) and progressive axonopathy in the Boxer (Griffiths, 1989).

1.3 Anatomy of the spinal cord structures involved in CDRM

In this section the structure of the spinal cord with the emphasis on areas which are of relevance to CDRM will be considered. Figure 1 (page 15) illustrates the spinal cord tracts of interest in CDRM. Most of the work done to date had been in the cat.

1.3.1 White matter

The white matter is divided into bilaterally symmetrical areas or funiculi in which most fibres are arranged in functional units or tracts. The long ascending tracts are essentially sensory while the long descending tracts contain the axons of the UMNs. The long descending tracts were previously referred to as the "motor" system, but this would be inaccurate as among other things they regulate the transmission of sensory impulses in the ascending tracts. White matter tracts are often represented as being well circumscribed and functionally discrete, but even in man this is not the case. In addition it is not appropriate to directly transfer detailed knowledge of human anatomy to other species.

1.3.2 Dorsal funiculus

The dorsal funiculus extends from the dorsal median sulcus to the entrance of the dorsal spinal nerve roots in the dorsolateral sulcus, it is made up of two large ascending fibre tracts, the fasciculus gracilis and fasciculus cuneatus. The fasciculus cuneatus is present only in the cervical and parts of the thoracic cord. These tracts are associated with conscious proprioception and contain fibres which enter the dorsolateral sulcus, pass over the dorsal horn and ascend in the ipsilateral dorsal funiculus. The fibres of these tracts are the longest in the body, they are large and heavily myelinated with high metabolic requirements.

1.3.2.1 Fasciculus gracilis

The fasciculus gracilis is the medial tract which borders the dorsal median sulcus and extends the whole length of the cord. This tract is mainly composed of axons from the cell bodies in the SpG of the nerves which innervate the pelvic limbs and caudal trunk. The arrangement of fibres is such that those fibres which enter the cord caudally lie medially within the cord, while fibres added as far forward as the mid-thoracic level lie laterally.

1.3.2.2 Fasciculus cuneatus

The fasciculus cuneatus lies lateral to the fasciculus gracilis and transmits impulses from the thoracic limb, cranial trunk and neck. Similarly to the fasciculus gracilis,

this tract is made up of axons from cell bodies in the SpG which enter the dorsal funiculus directly *i.e.* without synapses. Again the arrangement of fibres is such that the longer fibres, this time from the mid-thoracic levels, lie medial to the shorter ones from the thoracic limb and cervical regions.

Both of these tracts convey impulses to the brain, the first neurone terminating in the respective nucleus (nucleus gracilis and nucleus cuneatus) in the medulla oblongata.

1.3.3 Lateral funiculus

The lateral funiculus lies between the dorsal and ventral spinal nerve roots and contains both ascending and descending tracts.

1.3.3.1 Ascending tracts

The ascending tracts usually lie around the peripheral margin of each spinal cord segment.

1.3.3.2 Dorsal spinocerebellar tract

The dorsal spinocerebellar tract (DSCT), also called Flechsig's fasciculus, is composed of heavily myelinated fibres whose cell bodies are within the thoracic nucleus (dorsal nucleus of Clarke). In the cat this extends from the cranial thoracic segments as far caudally as the fourth lumbar segment (Hubbard and Oscarsson, 1962). The axons pass ipsilaterally from this nucleus to lie at the periphery of the cord between the dorsal root entrance and the lateral corticospinal tract. Caudally the tract begins at segments L3 or L4 and increases in size as it passes cranially throughout the levels of the thoracic nuclear column (this region has been accurately defined in the cat as extending from L3 to T3). The DSCT enters the cerebellum *via* the caudal cerebellar peduncle. Verhaart and van Beusekom (1958) reported that this tract contained a mixture of fibres from the pyramidal and other tracts. They also reported that not all fibres reach the cerebellum, some terminating in the lateral cervical nucleus of the cord, others in the medullary nuclei. The DSCT is involved with the unconscious subdivision of proprioception transmitting impulses from neuromuscular spindles, Golgi tendon organs, touch and pressure receptors in the skin of the pelvic limbs and caudal trunk to the cerebellum. The cerebellum then exerts its tonic and synergising influence on the motor pathways. Although the proprioceptive neurones are sensory (general somatic afferent) their dysfunction is recognised clinically by assessing motor performance. The important sign associated with damage to these tracts is ataxia (dyssynergia).

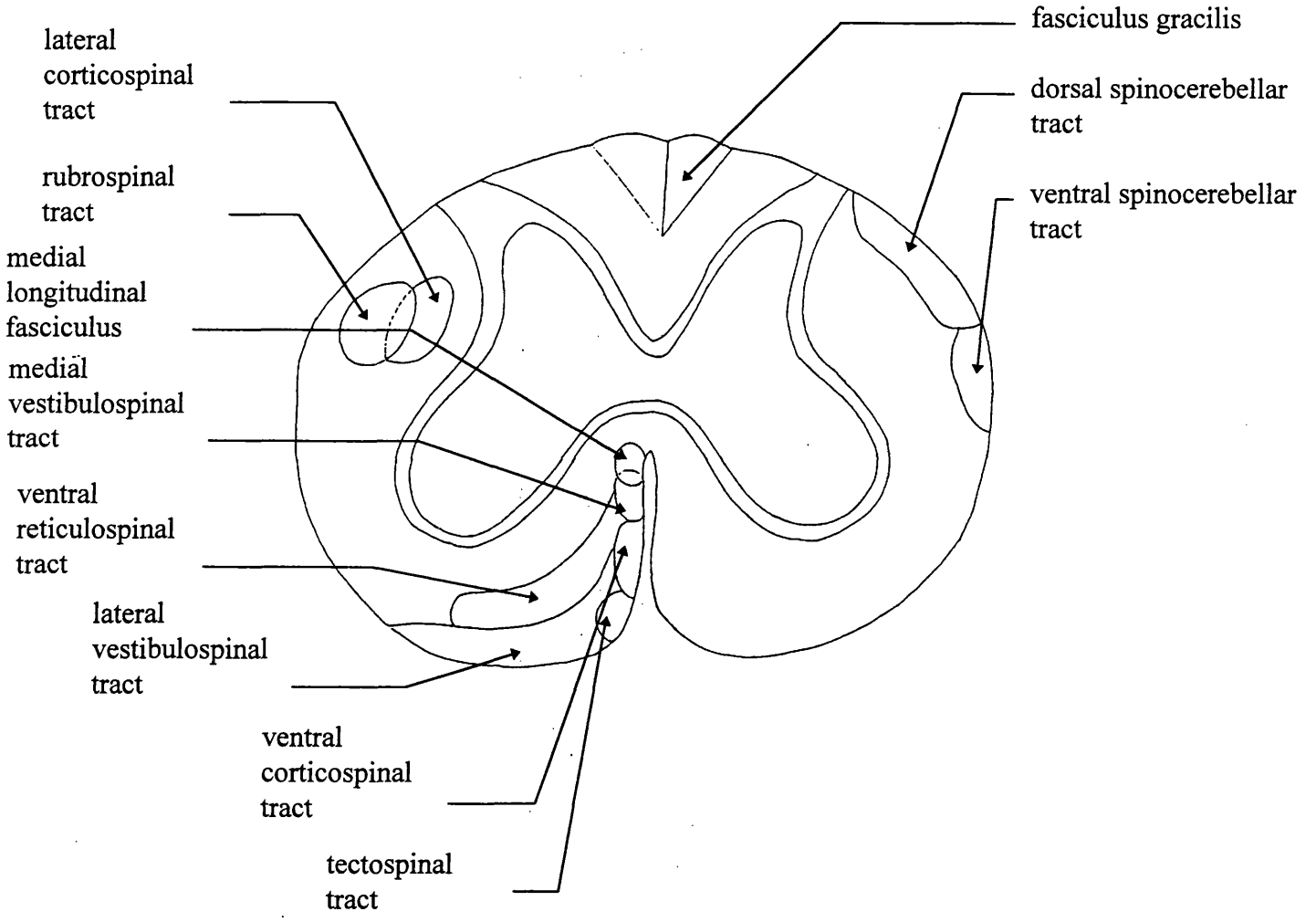


Figure 1. Canine cervical spinal cord segment to show the relevant descending tracts (left side) and ascending tracts (right side) which are affected in CDRM. Adapted from Jenkins (1978).

1.3.3.3 Ventral spinocerebellar tract

The ventral spinocerebellar tract lies at the periphery of the lateral funiculus ventral to the DSCT and is bordered medially by the lateral spinothalamic tract. The origin is diffuse, although in the cat the majority of the cells of origin lie in the lateral base and neck of the dorsal horn in the lumbar segments, with a few in adjacent parts of the ventral horn and the head of the dorsal horn (Hubbard and Oscarsson, 1962). This tract transmits only impulses which arise in the pelvic limbs and caudal trunk. Some axons remain ipsilateral while others decussate then decussate back again at the level of the cerebellum. In the cat the position of this tract varies as it passes cranially; moving dorsally from the lumbosacral through the thoracic and caudal cervical segments, then ventrally in the cranial cervical segments (Grant, 1962). Ultimately this tract ascends through the brain stem to the peripheral margin of the rostral cerebellar peduncle which it follows into the cerebellum. The fibres transmit impulses generated from large receptive fields of Golgi tendon organs and flexor reflex afferents (Noback and Demarest, 1986). The ultimate projection of the ventral spinocerebellar tract is ipsilateral.

1.3.3.4 Descending tracts

The descending tracts lie between the grey matter and the ascending tracts. These are upper motor neurones (UMNs) which terminate by entering the grey matter to synapse with an interneuron, thus indirectly synapsing with the cell body of lower motor neurones (LMNs).

1.3.3.5 Corticospinal tracts

Fibres of the lateral corticospinal tract originate throughout the cerebral cortex and descend through the ipsilateral brain stem to the spinomedullary junction. This is the major pathway by which the cerebral motor cortex connects directly to the spinal cord (Buxton and Goodman, 1967). At the level of the spinomedullary junction many of the fibres, 75-85%, cross in the pyramidal decussation and descend as far caudally as the lumbar segments in the dog. The remaining uncrossed fibres, 15-25%, descend in the ipsilateral ventral funiculus as the ventral corticospinal tract. In veterinary neuroanatomy the lateral corticospinal tract is functionally insignificant, in contrast to its great importance in man. Dysfunction of this tract in man, which commonly occurs following strokes, causes a severe spastic hemiparesis. The location of terminal fibre synapses is the factor which determines to what extent the corticospinal tract directly influences movement. In man the fibres synapse directly on LMNs, so directly influence movement. In animals the

corticospinal pathways terminate in the dorsal horns and synapse on interneurons thus indirectly influencing the action of the LMNs. Damage to these tracts in the dog would result in hypotonia, hypokinesia and dysmetria on the contralateral side. These functional deficits would be more severe in the hindlimbs than the thoracic limbs.

1.3.3.6 Rubrospinal tract

The rubrospinal tract, also called von Monakow's bundle, arises from cells throughout the red nucleus (for details see page 20) which lies in the midbrain tegmentum. Much work has been done on the possible somatotopic arrangement of this nucleus in the cat (Pompeiano and Brodal, 1958; Nyberg-Hansen and Brodal, 1964a). Fibres serving the lumbosacral cord originate in the ventral and ventrolateral regions of the nucleus, those serving the thoracic cord originate in the intermediate portion and those destined for the cervical cord arise in the dorsal and dorsomedial portions of the nucleus. On leaving the red nucleus, fibres immediately cross the median raphe as the ventral tegmental decussation and descend in the lateral brain stem tegmentum then continue in the lateral funiculus of the spinal cord. Due to their location, the rubrospinal fibres partly intermingle with the descending fibres of the lateral corticospinal tract, although the majority lie ventrolateral to this tract. In the dog the rubrospinal tract has been traced throughout its length, as far caudally as the lumbar segments whereas in the cat a few fibres extend to the sacral segments (Hinman and Carpenter, 1959; Staal and Verhaart, 1963). It has been reported that most of the fibres are received by the cervical cord segments, with the remainder going to the thoracic and lumbar segments (Pompeiano and Brodal, 1958). The rubrospinal fibres enter the spinal cord grey matter at the level of the lateral horn terminating in the fifth, sixth and seventh laminae (Nyberg-Hansen and Brodal, 1964a). The actual termination points for these fibres are subject to some controversy for two reasons; firstly, most experimenters used the Marchi technique which does not demonstrate terminal ramifications of fibres and secondly, the terminology used to describe the different grey matter zones is vague and confusing. The majority of the rubrospinal fibres terminate in the intermediate grey matter (de LaHunta, 1977). This tract is of vital importance in animals, more so than in man, because it is concerned with coordination and possibly also locomotion. The rubrospinal tract exerts a number of influences on the spinal cord. Through interneurons it excites alpha and also gamma motoneurons of the contralateral flexor muscles. At the same time, it inhibits the alpha fibres and the static fusimotor fibres of the contralateral extensor muscles. It also facilitates a set of inhibitory and excitatory spinal reflexes by direct action on relevant interneurons. The rubrospinal tract is responsible for the

facilitation of the cells of origin of the ventral spinocerebellar tract and, by means of presynaptic inhibition, controls activity in the primary afferents as these enter the spinal cord.

1.3.4 Ventral funiculus

The ventral funiculus lies between the emerging ventral spinal nerves and the ventral median fissure and contains mainly descending tracts. The tracts in this region are generally smaller and more dispersed, tending to overlap more than in other funiculi.

1.3.4.1 Descending tracts

1.3.4.2 Tectospinal tract

The tectospinal tract arises mainly from the rostral colliculus of the midbrain tectum. The fibres cross over and descend in the ventral funiculus bordering the ventral median fissure. Most of the fibres terminate in the upper four cervical segments by synapsing with internuncial neurones to the LMNs. Specific functions for this tract are difficult to define but, in combination with interstitiospinal fibres and the medial vestibulospinal tract, it is involved in reflex postural movements of the eyes, head and body.

1.3.4.3 Ventral corticospinal tract

The ventral corticospinal tract has been considered with the lateral corticospinal tract see 1.3.3.5 Corticospinal tracts (page 16).

1.3.4.4 Lateral vestibulospinal tract

The lateral vestibulospinal tract in the cat contains descending fibres from the ipsilateral lateral vestibular nucleus (Nyberg-Hansen and Mascitti, 1964b). In the dog the pathway extends to the lumbosacral cord with the cervical and lumbar enlargements receiving more fibres than the grey matter of the thoracic segments. A somatotopic organisation has been recognised in the vestibulospinal projection, such that the pelvic limb region of the cord is served by the dorsocaudal region of the lateral nucleus (Nyberg-Hansen and Mascitti, 1964b). The lateral vestibulospinal tract functions to maintain equilibrium and is of great importance in all carnivores. The medial vestibulospinal tract arises from the medial vestibular nucleus and descends bilaterally, but mainly ipsilaterally, within the medial area of the ventral funiculus. This tract is much less important than its lateral counterpart and extends only as far as the mid-thoracic spinal cord segments.

1.3.4.5 Medial longitudinal fasciculus (MLF)

The medial longitudinal fasciculus (MLF) which consists of both ascending and descending fibres, extends from the level of the rostral midbrain caudally throughout the brain stem then continues into the spinal cord ventral funiculus. In the dog this tract passes caudally to lumbar levels. Functionally, the MLF acts as a connector between vestibular and motor nuclei of cranial nerves III, IV and VI accounting for vestibular reflexes involving eye movements. Due to the large size of this tract and its ventral position in domestic animals, it is prone to injury.

1.3.4.6 Ventral reticulospinal tract

The ventral reticulospinal tract is less often involved in cases of CDRM than the previously described, lateral vestibulospinal tract. The reticulospinal fibre system is complex and is described differently by various authors. The ventral reticulospinal tract is thought to arise from the caudal pontine reticular area, the fibres then descend almost exclusively on the same side within the ventral funiculus. They continue as far caudally as the lumbosacral segments (Nyberg-Hansen, 1966). This pontine (as opposed to medullary) reticulospinal tract, along with the lateral vestibulospinal tract, facilitates extensor motoneurones. Unlike the rubrospinal and corticospinal tracts there is no evidence for somatotopic organisation in this tract.

1.3.5 Grey matter

The grey matter is made up mainly of neurone cell bodies, myelinated and non-myelinated fibres, glial cells and capillaries. The supporting neuropil is formed from neuronal dendrites and axons plus a fine meshwork of glial processes. Rexed (1952; 1954) has subdivided the grey matter of the cat into ten distinct laminae, based on differential cytoarchitectonics. This system provides a precise criterion for localization of specific axon terminations and origins. Romanes (1951) correlated specific cell bodies in the ventral horn of the grey matter of the cat spinal cord with innervation of individual muscles in the pelvic limb. This level of knowledge is still not available for the dog. Essentially, the grey matter is considered to consist of several regions; the main ones being dorsal horn, lateral horn, ventral horn and Clarke's column. The region of particular interest in CDRM is the area of grey matter level with the central canal and lying between the dorsal and ventral horns. The classification system used in this thesis is that of de Lahunta (1977) who designated this area the intermediate grey horn.

1.3.5.1 Dorsal horn (column)

This region of the grey matter is infrequently involved in CDRM. It is made up of a number of nuclear groups, namely the substantia gelatinosa, nucleus proprius, secondary visceral nucleus, thoracic nucleus and lateral cervical nucleus. The thoracic nucleus lies dorsolateral to the central canal in the thoracic and cranial lumbar portions of the cord. This nucleus is the origin of the dorsal spinocerebellar tract.

1.3.5.2 Intermediate horn (column)

This is the region of grey matter most often affected in CDRM. The general somatic afferent and general proprioception neurones are located in this region with the general somatic efferent neurones.

1.3.5.3 Lateral horn (column)

A small evagination of grey matter which is visible from T1 caudally, lying just lateral to the level of the central canal. This region indicates the stratum of cell bodies which are the nucleus of origin for the general visceral efferent sympathetic neurones.

1.3.5.4 Ventral horn (column)

This area contains the large multipolar cell bodies of the alpha motor neurones. In addition, the ventral horn also contains the smaller cell bodies of the gamma efferent neurones which transmit impulses to motor endings of the intrafusal muscle fibres in the neuromuscular spindles. These are both examples of LMNs. These LMN cell bodies are found throughout the entire length of the spinal cord.

1.4 Anatomy of the brain nuclei involved in CDRM

Again, most of the anatomical and physiological investigations have been carried out in the cat.

1.4.1 Red nucleus

This nucleus lies in the tegmentum at the level of the rostral colliculus, embedded within the reticular formation and ventral to the oculomotor nucleus. It can be seen on gross examination of the sectioned brain due to its distinct red colour which is thought to be due to the high level of vascularisation within the nucleus. Identification is also aided by the presence of a distinct "capsule" formed by the

fibres of the rostral cerebellar peduncle (brachium conjunctivum) (Hinman and Carpenter, 1959).

At a cellular level, the red nucleus is composed of a number of large, medium and small cells. In man these can easily be separated into a caudal magnocellular and a rostral parvocellular population. However, this distinction in cell sizes is much less well defined for carnivores than other mammals. All the efferents from the magnocellular part of the red nucleus are crossed. The main efferent pathway is the rubrospinal tract (for details see page 17), other efferents pass to the bulbar lateral reticular nucleus, the facial nucleus and the cerebellar nucleus interpositus. The cells within this tract are somatotopically arranged, the pelvic limb being served by the ventrolateral region while the thoracic limb is served by the dorsomedial region. A similar topographic arrangement is found among the corticorubral and cerebellorubral projections. The parvocellular region of the red nucleus contains mainly small and medium sized cells and, in carnivores only, a small number of large cells. Efferent connections to this part of the nucleus are ipsilateral, consisting of rubrothalamic and rubro-olivary fibres. Most of the afferent fibres originate from the contralateral nucleus interpositus.

The red nucleus contains colonies of neurones which ultimately activate the motoneurone pools of individual muscles. These effects are mediated by particular interneurones in the dorsolateral interneuronal cell groups of the spinal cord. There is considerable overlap of individual rubral efferent colonies. Microstimulation within the red nucleus is capable of eliciting contraction of both flexor and extensor muscles of the contralateral limb (Ghez, 1975).

1.4.1.1 Origin of afferent fibres

Afferent fibres arise in the opposite side of the cerebellum via the rostral cerebellar peduncle which enters the tegmentum and crosses caudal to the red nucleus as the decussation of the brachium conjunctivum. Most of these cerebellorubral fibres originate in the nucleus interpositus with a small number of fibres originating in the lateral nucleus. Fibres from the most rostral part of the nucleus interpositus pass to the caudal two thirds of the red nucleus which in turn sends fibres to the contralateral pelvic limb. Projections from the caudal part of the nucleus interpositus end in the area of the red nucleus which influences the thoracic limb. Afferent fibres also arise from the cerebrum and corpus striatum (Jenkins, 1978).

1.4.1.2 Projections

Unlike other nuclei in the reticular formation, efferent fibres of the red nucleus project into spinal segments more caudal than the thoracic segments and in addition, parts of the nucleus project large fibre bundles to brain stem nuclei, the cerebellum and the diencephalon.

The principal efferent pathway is the rubrospinal pathway which has been discussed on page 17. A number of other efferent pathways exist. The rubrocerebellar tract (Massion, 1967) has been recognised in both rodents and carnivores. Fibres originate mainly from the large neurones in the caudal third of the contralateral red nucleus with most fibres ending in the nucleus interpositus. The presence of the rubro-olivary tract whose ipsilateral fibres originate in the rostral red nucleus and terminate in the dorsal lamina of the principle olive has also been confirmed (Hinman and Carpenter, 1959). Other efferent connections have been described, ipsilateral rubropallidal and rubrosubthalamic fibres have been identified in a number of species, including the cat. Both direct and crossed rubrotegmental fibres have been described by von Monakow, Weisschedel (both in German) and Rioch (1929). Rubrobulbar fibres fall into two categories; the first being the uncrossed fibres which pass to the dorsal lamellae of the principal inferior olivary nucleus; the second being crossed fibres passing to specific portions of the lateral reticular nucleus of the medulla which arise from the caudal two thirds of the contralateral red nucleus.

Finally, rubrorubral connections passing *via* the caudal commissure and a tract connecting the red nucleus to the contralateral hypothalamus have been reported.

1.4.2 Vestibular nuclei

The vestibular system consists of a group of four nuclei *i.e.* lateral, medial, rostral and caudal, on each side of the midline in the floor of the fourth ventricle at the level of the upper medullary-pons junction. The lateral vestibular nucleus (LVN) or nucleus of Deiters is the one most commonly affected in CDRM. This nucleus lies immediately rostral to the spinal nucleus and is medial to the caudal cerebellar peduncle and dorsomedial to the spinal tract and nucleus of the trigeminal nerve. The LVN is generally defined as the part of the vestibular complex containing giant cells of Deiters, although it also contains a number of medium and small sized cells (Nyberg-Hansen and Mascitti, 1964b). The giant cells are generally present in greater numbers in the dorsocaudal part of the nucleus, while the medium and small cells tend to be in the rostral part. The axons of all these cells pass to the spinal cord in the vestibulospinal tract. The medial (Schwalbe's) vestibular nucleus lies medial to the LVN and dorsolateral to the MLF.

1.4.2.1 Origin of afferent fibres

Direct primary vestibular axons from the vestibular (Scarpa's) ganglion enter all four nuclei via the vestibular nerve. Cerebellovestibular fibres from the cerebellar cortex and fastigial nucleus provide a feedback mechanism between the vestibular nuclei and the cerebellum. The general proprioceptive impulses from the neck muscles, tendons and joints permit head movements and position to direct the orientation of the body in space as the primary basic principle in the vestibular righting reflex.

1.4.2.2 Projections

Fibre connections from the vestibular nuclei can be separated into two main pathways. In the first of these, descending axons from the lateral vestibular nucleus project ipsilaterally as the lateral vestibulospinal tract (the tract is exclusively uncrossed) to the ventral funiculus of the lumbosacral spinal cord in the dog (Nyberg-Hansen and Mascitti, 1964b). The vestibulospinal projection has a somatotopic organisation, the pelvic limb being served by the dorsocaudal region. In the second pathway axons originating from all four vestibular nuclei pass medially to enter the MLF. In addition, a group of secondary vestibulocerebellar axons are relayed from the various nuclei to the cerebellar cortex.

1.4.2.3 Functions

The LVN, by way of the vestibulospinal tract (Pompeiano and Brodal, 1957), exerts a facilitatory effect on spinal reflexes which control muscle tone, especially to maintain appropriate posture or strength of supporting and balancing movements. Maintenance of equilibrium is largely reflex activity governed primarily by general proprioceptive impulses from muscles, tendons and joints in the trunk and limbs and special proprioceptive nerve endings in the labyrinth which initiate impulses conveyed *via* the vestibular nerve to the vestibular nuclei. The vestibulospinal tracts (along with the reticulospinal tracts) have a facilitatory effect on the spinal cord LMNs especially those supplying the antigravity (*i.e.* the postural and extensor) muscles. A failure in the normal activity of the vestibular system due to labyrinthine overstimulation, disease, irritative or destructive lesions may cause disequilibrium, staggering, postural changes, falling or rolling to the same side, nystagmus, deviation of the eyes, nausea, vomiting and vasomotor reactions.

1.4.3 Lateral (dentate) nucleus

The lateral nucleus is one of the three cerebellar nuclei which are embedded in the white matter dorsal to the fourth ventricle, the other two being the fastigial nucleus

and the nucleus interpositus. The lateral nucleus is the most lateral of the three but is difficult to differentiate anatomically from the neighbouring nucleus interpositus. This nucleus has a dorsal and a ventral eminence protruding laterally to give a tooth-like shape which is less pronounced in the dog than in other species.

1.4.3.1 Origin of afferent fibres

Afferent fibres originate from the rostral and caudal lobes of the cerebellum *via* the axons of the Purkinje cells.

1.4.3.2 Projections

Fibres project to the red nucleus, the reticular formation, the pallidum and the ventrolateral nucleus of the thalamus through the rostral cerebellar peduncle.

1.4.3.3 Function

The lateral nucleus is concerned with regulation of skilled, spatially organised movements of the limbs, but has no direct regulatory function on posture or tone (Holliday, 1979). In addition, recent medical research using MRI has suggested that the lateral nucleus may be active during motor, perceptual and cognitive performances due to the requirement to process sensory data (Nyberg-Hansen, 1966; Barinaga, 1996).

1.5 Comparable Diseases In Other Species

1.5.1 Feline

Degenerative myelopathy had occurred very infrequently in the cat, being reported only in two cats infected with Feline leukaemia virus (FeLV) (Mesfin *et al.*, 1980). The pathology of the one case available was dissimilar to CDRM.

1.5.2 Equine

Equine degenerative myeloencephalopathy (EDM) had occurred not only in domesticated horse breeds such as Appaloosas, Arabians, Quarter horses, Thoroughbreds, Standardbreds and Morgan horses, but also in the Prezwalski horse and the zebra (Dill *et al.*, 1989; Dill *et al.*, 1990; Blythe and Craig, 1992a; Blythe and Craig, 1992b) and was characterised clinically by ataxia, especially of the pelvic limbs, and a wide-based stance and gait. A number of affected animals also had laryngeal adductor, cervicofacial, local cervical and cutaneous trunci hyporeflexia (Mayhew *et al.*, 1987). Clinical signs usually developed within the first twelve

months of life. This condition was most common in the northeast United States but had been seen in other parts of North America, the United Kingdom (Whitwell, 1980) and in two Haflinger fillies in Germany (Baumgartner *et al.*, 1990). There was no obvious sex predisposition. On histopathological examination of the spinal cord there was diffuse degeneration of both axons and myelin sheaths, the ventromedial and dorsolateral funiculi were the most consistently affected. Changes included the presence of spheroids (axonal swellings), neuronal atrophy, astrogliosis and accumulation of lipofuscin-like pigment in neurones. Grey matter changes were seen in the nucleus of the spinocerebellar tracts in the thoracic region and in several nuclei of the lateral horn. These grey matter changes were reported in detail by Mayhew (1977; 1987). Brain changes involved neuroaxonal dystrophy in brain stem nuclei, especially the medial and lateral cuneate nuclei and the nucleus gracilis. Neuroaxonal dystrophy was characterised by the presence of disseminated axonal swellings (spheroids) in preterminal portions of axons and in synaptic terminals.

A condition called degenerative myelopathy had been reported in a small group of Prezwalski horses (Liu *et al.*, 1983), a Welsh pony and a donkey (Scarratt *et al.*, 1985). The clinical signs and spinal cord pathology had marked similarities to EDM. The brain was not examined at post mortem in these cases. The available evidence would suggest that these cases all represented the same condition.

The pathogenesis for this condition(s) was not understood but there was a body of evidence which suggested that vitamin E may have been involved. Firstly, experimentally induced vitamin E deficiency in rats (Southam *et al.*, 1991) and rhesus monkeys (Nelson *et al.*, 1981) often caused a neuroaxonal dystrophy with lipofuscin-like pigment accumulation, as had been noted in the aforementioned equine cases. Secondly, a naturally occurring vitamin E deficiency in humans resulted in neuroaxonal dystrophy with the same pigment accumulation. In addition, inadequate serum or plasma ∝-tocopherol concentrations had been reported in several studies on EDM. However, unaffected horses from different lines had equally low concentrations of serum α-tocopherol. Prophylactic administration of d,1 ∝-tocopherol, at a level of 1000 to 2000IU/day, to foals from sires known to produce EDM-affected offspring led to a decrease in disease incidence. In the first year the incidence dropped from 40% to 10% (Mayhew *et al.*, 1987) and subsequently from 10% to 1% over the next three years. In one study (Blythe and Craig, 1992b) affected horses were treated with 6000IU/day of d,1-∝-tocopherol acetate for several years. The neurological scores improved in all cases.

The pathology seen in these equine diseases had similarities to CDRM, both in the distribution of the lesions within the spinal cord *i.e.* predominantly the dorsolateral

and ventromedian regions of the white matter and in the nature of the changes seen *i.e.* degeneration of both axons and myelin sheaths. However, degeneration in the grey matter was much more marked than in CDRM which suggested that underlying pathogenetic mechanisms were unlikely to be exactly the same.

1.5.3 Bovine

A number of neurologic disorders with some similarities to CDRM had been reported in several breeds of cattle. Bovine progressive degenerative myeloencephalopathy (BPDME) had been seen in Brown Swiss cattle in Canada, Denmark, Germany, Switzerland and the United States of America (Leipold *et al.*, 1973; Stuart and Leipold, 1983; Stuart and Leipold, 1985; Baird *et al.*, 1988; Oyster *et al.*, 1991a; Oyster *et al.*, 1991b). This condition was characterised clinically by a progressive pelvic limb weakness and ataxia which usually began at five to eight months of age and resulted in permanent recumbency over a 12 to 18 month period. On microscopic examination of the CNS the spinal cord white matter was the most consistently and severely affected area. Active axonal degeneration, loss of axons and myelin, status spongiosus and very large axonal swellings were visible at all levels of the cord. The degenerate axons were present in both ascending and descending tracts of the white matter and were considered to be the primary lesions of BPDME. The thoracic region and the ventral funiculi were the most severely affected regions in all cases. No significant changes were found in the grey matter, spinal motor neurones or SpG at any level.

In the brain, spheroids and pyknotic glial cells were seen in the subcortical white matter of the cerebrum, cerebellum and medullary pyramids. Lesions in the grey matter of the brain were found in the olivary nucleus of the medulla oblongata and consisted of degenerative changes which ranged from neuronal vacuolar degeneration to chromatolysis, accompanied by mild, reactive astrocytosis. In addition, oligodendroglia numbers were reduced. Although the pathogenesis was not understood, BPDME was considered to be a degenerative spinocerebellar disease with familial predisposition.

A progressive spinal myelinopathy had been seen in Murray grey cattle in Western Australia (Richards and Edwards, 1986). The age of onset was most commonly within the first six months of life. This condition was characterised clinically by spinal ataxia manifest as pelvic limb incoordination and a lateral swaying of the hindquarters when standing. These animals also exhibited an increased muscle tone in the pelvic limbs. At post mortem, microscopic lesions were found in the spinal cord white matter of all cases but only the ventral and lateral funiculi were consistently affected. The lesions were bilaterally symmetrical, present throughout

the length of the cord and generally affected all segments equally. Peripheral white matter tracts were usually severely, but diffusely, affected with relative sparing of the propriospinal tract. Several cases had shown dorsal funicular lesions which affected both the fasciculus gracilis and fasciculus cuneatus. The pathology consisted of numerous dilated myelin sheaths, many of which still contained axons of normal diameter. Swollen axons were present in a small proportion of these fibres while some sheaths contained mononuclear macrophages (gitter cells). Occasionally, "digestion chambers" were seen, made up of macrophages and cell debris, which were typical of Wallerian degeneration. Glial cell (cell type not specified) density was slightly increased within the spinal lesions. Spinal nerve roots were unaffected. A few chromatolytic neurones were present in all spinal cord segments, usually in the grey matter of the ventral horn but occasionally in the thoracic nucleus. There did not appear to be an overt neuronal dropout in the spinal cord grey matter.

In the brains of most affected calves, white matter degeneration in the spinocerebellar and tectospinal tracts and the MLF was noted. These lesions resembled the spinal cord white matter changes. Chromatolytic neurones were often present in the red nucleus of the midbrain, LVN, medial portion of the reticular formation (brain region not specified) and occasionally in the cerebellar nuclei. This chromatolysis was usually diffuse and involved some swelling of the cell body, however no cellular necrosis was observed.

Both these conditions in cattle show many clinical and pathological similarities to CDRM although one marked difference is the early age of onset in the affected cattle.

1.5.4 Other herbivores

Ataxia associated with spinal cord degeneration had been seen in a number of other species such as llama, wildebeeste and camel (Palmer *et al.*, 1980). There have been several reports of a pelvic limb ataxia and paresis occurring in the llama (Palmer *et al.*, 1980; Morin *et al.*, 1994). Clinically, the affected llamas showed a variety of different pictures which varied from a unilateral pelvic limb ataxia and paresis (Palmer *et al.*, 1980) through to sudden onset recumbency (Morin *et al.*, 1994). No gross lesions were found in any of the affected animals and on microscopic examination of the CNS very similar patterns of degeneration were seen in all affected animals. In the cord, bilateral axonal degeneration was found throughout all spinal cord segments, this was particularly severe in the thoracic cord. The tracts most severely affected were the dorsal and dorsolateral sensory tracts, only minimal degeneration was visible in the cervical cord. The changes

consisted of myelin sheaths which contained cellular debris and gitter cells, the latter often contained lipofuscin granules. Grey matter changes were minimal and confined to the ventral horn, they consisted of a few distended eosinophilic structures which were interpreted to be swollen, dystrophic axons (spheroids). In some brain stem nuclei small numbers of hyperchromatic and slightly shrunken degenerate neurones, often containing lipofuscin were observed. No evidence of inflammatory change or specific pathogens was observed.

There had been one isolated case of ataxia reported in a one year old camel (Palmer *et al.*, 1980), in which degenerative changes resembled those seen in the llamas. Ataxic wildebeeste have been examined post mortem (Palmer *et al.*, 1980) and Wallerian degeneration of ventral and lateral funiculi, especially spinocerebellar tracts was noted. The degeneration was traced up into the caudal cerebellar peduncle.

To date a number of conditions with some similarities to CDRM have been reported in several other species but minimal attempt had been made to study the aetiopathogenesis. The obvious exception to this were the human neurodegenerative disorders. A number of diseases with clinical and pathological similarities to CDRM occurred in this species. Of particular interest were a group of late onset, progressive neurodegenerative diseases which resulted in selective loss of neurone populations. These were the result of CAG trinucleotide repeat expansions. Several of these conditions have recently been investigated such that the actual gene defect has been characterised. These conditions are considered in detail in 6 An investigation towards the identification of a possible molecular lesion associated with CDRM (page 159).

1.6 Aims of the thesis

Since 1973 a progressive, neurodegenerative disease had been recognised in large breed dogs with a higher incidence in the GSD than other breeds. A number of aetiological factors had been suggested, including various vitamin deficiencies, or immune-mediated disease, but none had been proven to be the cause of CDRM. Most authors considered a genetic factor likely due to the high incidence of the disease in one breed. The clinical signs occurred in older GSDs and involved a progressive pelvic limb ataxia and paresis. Local spinal reflexes in the pelvic limbs were commonly intact, although the patellar reflex varied in response to the disease. The perineal and panniculus reflexes were unaffected. Clinical signs progressed over a six to 12 month period before the dogs lost useful pelvic limb function. There was no diagnostic test available for use in the live animal. Diagnosis was

dependent on pathological examination of the spinal cord. Only one author had suggested an effective treatment regime which included vitamin E, vitamin B and EACA, this was not confirmed by other workers in the field. CDRM was still an untreatable disease.

The pathology in the spinal cord had been described in detail by a number of authors. Most agreed that the white matter areas of the spinal cord were the most consistently affected, with degeneration which involved both axons and myelin, often with an associated gliosis. The caudal thoracic segments were consistently the most severely affected. The grey matter changes reported varied from loss of neurones in the ventral horn (Averill, 1973) to increased astrocyte numbers in the dorsal and lateral horns (Griffiths and Duncan, 1975a). Only one author suggested that there was evidence of axonal regeneration in the spinal cord (Clemmons, 1989), the same author mentioned the possibility of changes occurring in unspecified white matter areas of the brain. A number of comparable conditions characterised by progressive pelvic limb ataxia and paresis, both in other breeds of dog and in other species had been described. As a general rule the pathology had been described in detail for most of these conditions but the aetiology was still unknown. There were obvious exceptions to this, most importantly in many of the human ataxias, where the underlying lesion was known to be genetic while in some the actual gene defect had been characterised.

This project aimed to examine clinically and pathologically a large number of GSDs affected by CDRM. Clinically, we wished to confirm the previously reported clinical signs, and look for any that may not have been reported. We proposed to follow the dogs through the clinical course of the disease, re-examining on a regular basis to establish whether the rate of degeneration was constant or variable. If variable, were there a number of recognisable patterns which might suggest we were dealing with a syndrome rather than a single disease ? In addition, we wished to confirm whether or not GSDs with CDRM had lower serum vitamin E concentrations than other dogs, since this vitamin had been associated with a number of neurodegenerative diseases in other species. The initial aim of the pathology investigation was to carefully examine the spinal cord using immunocytochemistry and electron microscopy, as well as classical techniques, to confirm the pathology previously reported and to look for new clues to the pathogenesis and aetiology using the more modern techniques. The next step was to carefully search through the brain, in particular those structures which, on the basis of the clinical signs, were most likely to be involved in CDRM. This search resulted in the discovery that specific brain nuclei and areas of white matter were altered in dogs with CDRM. These findings suggested marked clinical and

pathological similarities between CDRM and a group of late onset progressive human neurodegenerative conditions which were the result of CAG trinucleotide expansions. This led into an investigation of the hypothesis that "CDRM is the result of a CAG trinucleotide repeat expansion".

2. Materials and Methods

2.1 COLLECTION OF CLINICAL MATERIAL

2.2 Case Selection

A full case history was obtained from the owner and referring veterinary surgeon and the relevant details were recorded. All cases underwent full systemic examination with only significant abnormal findings being recorded.

All cases received a complete neurological examination as detailed by Oliver and Mayhew (1987). Mentation and posture were noted and the owners questioned about any change in behaviour or attitude. The gait was evaluated for lameness, coordination and weakness. Conscious proprioception and motor function were assessed by paw position, reflex stepping, hip sway test and wheelbarrowing. The reflex step is a test of proprioception and is not a true reflex, this test can also be referred to as the "piece of paper test". The hip sway test was carried out by holding the dog either side of the midline in the flank region and gently moving the dog over to one side then the other, a dog with normal proprioception will immediately move the relevant foot such that the body is properly supported (Griffiths and Duncan, 1975a). Local spinal reflexes were assessed in each limb with the dog in lateral recumbency. Muscle tone was assessed by passive flexion and extension of the limb. The patellar reflex was assessed, but myotactic reflexes were not routinely carried out in the thoracic limbs as these were known to be inconsistent, even in animals with normal thoracic limb function (Dr C.E. Thomson, personal communication, 1995). The pedal reflex was tested to evaluate flexor muscle group strength and to assess cutaneous sensory fields in the distal limb. The presence or absence of conscious pain perception was noted. Muscle bulk and symmetry were assessed by palpation and the joints were examined for the presence of musculoskeletal disease. The panniculus reflex was tested on each side. Bladder and bowel function was evaluated by questioning the owner and examination of the perineal reflex.

Based on these findings an attempt was made to localise the lesion to the T3-L3 region of the spinal cord.

A complete list of all dogs included in the project, both CDRM cases and controls is given in Table 1, pages 33-44.

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
1	129202	GSDX	10	FN	G+	+	+	+	+
2	126438	GSD	12	FN	G+	+	+	+	+
3	126586	GSD	7	FN	G+	+	+	+	+
4	126496	GSD	8	M	G+	+	-	+	+
5	125636	GSD	9	M	G+	+	+	+	+
6	129449	GSD	9	M	G+	+	-	-	+
7	126403	GSD	12	F	G+	+	+	+	+
8	126063	GSD	9	M	G+	-	-	-	+
9	126126	GSD	8	FN	G+	+	-	-	+
10	125880	GSD	10	F	G+	-	-	-	+
11	129769	GSD	10	F	G+	+	+	+	+
12	127432	GSD	8	M	G+	+	+	-	+
13	114803	GSD	8	FN	G+	+	+	-	+
14	129800	GSD	9	M	G+	+	+	+	+
15	129450	GSD	9	FN	G+	+	-	-	+
16	129527	GSD	9	F	G+	+	+	-	+

Table 1a. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
17	126750	GSD	7	FN	G+	+	-	-	+
18	127398	GSD	8	M	G+	+	-	-	+
19	128951	GSD	9	M	G+	+	-	+	+
20	126497	GSD	9	M	G+	+	-	-	+
21	CDRM3	GSD	10	M	G+	+	-	-	+
22	129965	GSD	9	M	G+	+	+	-	+
23	CDRM4	GSD	9	M	R+	-	-	-	+
24	CDRM5	GSD	8	M	R+	-	-	-	+
25	CDRM6	GSD	7	F	R+	-	-	-	+
26	CDRM7	GSD	10	M	R+	-	-	-	+
27	CDRM8	GSD	9	F	R+	-	-	-	+
28	127996	GSD	11	M	G+	+	-	-	+
29	129644	GSD	9	M	G+	+	+	-	+
30	130538	GSD	8	M	G+	+	+	-	+
31	129966	GSD	9	M	G+	+	+	+	+
32	CDRM9	GSD	12	M	R+	-	-	-	+
33	130541	GSD	8	F	G+	+	+	+	+

Table 1b. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
34	CDRM10	GSD	10	MN	R+	-	+	-	+
35	CDRM11	GSD	8	M	R+	-	-	-	+
36	127994	GSD	12	M	G+	+	-	-	+
37	CDRM12	GSD	10	M	G+	-	-	-	+
38	CDRM13	GSD	8	F	R+	-	-	-	+
39	129625	GSD	9	FN	G+	+	+	-	+
40	131739	GSD	12	FN	G+	+	+	-	+
41	132560	GSD	8	M	G+	+	+	+	+
42	CDRM14	GSD	10	M	R+	-	-	-	+
43	132090	GSD	12	FN	G+	+	+	-	+
44	CDRM15	GSD	12	M	R+	-	-	-	+
45	CDRM16	GSD	8	F	R+	-	-	-	+
46	CDRM17	GSD	6	M	G+	-	+	+	+
47	132913	GSD	8	M	G+	+	+	+	+
48	CDRM18	GSD	8	F	R+	-	-	-	+
49	128291	GSDX	8	FN	G+	+	+	+	+
50	129057	GSDX	12	M	G+	+	-	+	+
51	130839	GSDX	8	M	G+	+	+	-	+

Table 1c. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
52	126755	GSD	9	M	G+	-	-	-	+
53	131613	GSD	8	M	G+	+	-	+	+
54	CDRM19	GSD	8	M	R+	-	-	-	+
55	126541	GSDX	9	F	G+	+	-	+	+
56	CDRM20	GSD	8	M	R+	-	-	-	+
57	CDRM21	GSD	8	M	R+	-	-	-	+
58	129238	GSD	8	M	G-	-	-	-	+
59	129237	GSD	8	M	G-	-	-	-	+
60	GSD1	GSD	3	F	G-	-	+	-	+
61	GSD2	GSD	3	FN	G-	-	+	-	+
62	GSD3	GSD	12	F	R-	-	-	-	+
63	128935	GSD	3	M	G-	-	+	-	+
64	GSD4	GSD	9	M	G-	-	+	-	+
65	GSD5	GSD	9	M	G-	-	-	-	+
66	GSD6	GSD	13	FN	G-	-	+	-	+
67	GSD7	GSD	9	F	G-	-	+	-	+
68	GSD8	GSD	10	M	R-	-	-	-	+

Table 1d. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
69	GSD9	GSD	12	F	R-	-	-	-	+
70	GSD10	GSD	10	F	R-	-	-	-	+
71	GSD11	GSD	9	FN	R-	-	-	-	+
72	GSD12	GSD	9	FN	G-	-	-	-	+
73	131707	GSD	11	M	G-	-	+	-	+
74	GSD13	GSD	9	MN	G-	-	-	-	+
75	GSD14	GSD	10	MN	G-	-	+	-	+
76	GSD15	GSD	10	F	R-	-	-	-	+
77	GSD16	GSD	8	F	R-	-	-	-	+
78	GSDX1	GSDX	8	FN	G-	-	-	-	+
79	GSDX2	GSDX	12	M	G-	-	+	-	+
80	GSD17	GSD	8	M	G-	-	+	-	+
81	125920	GSDX	9	FN	G-	-	+	-	+
82	GSD18	GSD	11	F	G-	-	-	-	+
83	Bob	Lab.	9	M	G-	-	+	-	+
84	Gean	Lab.	3	FN	G-	-	+	-	+
85	Zulu	Lab.	10	M	G-	-	+	-	+

Table 1e. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
86	Lewis	Lab.	1	F	G-	-	-	-	+
87	Aoifa	DH	6	FN	G-	-	-	-	+
88	Jack	JR	10	M	G-	-	+	-	+
89	Fanny	JR	10	FN	G-	-	+	-	+
90	Daz	JR	2	M	G-	-	+	-	+
91	Tanya	BC	15	FN	G-	-	+	-	+
92	Megan	BC	8	FN	G-	-	-	-	+
93	Jenny	Collie X	1	FN	G-	-	-	-	+
94	Rudi	Collie X	8	FN	G-	-	+	-	+
95	Blackie	Collie X	8	M	G-	-	-	-	+
96	Bronwyn	FCR	7	F	G-	-	+	-	+
97	Rosco	Lurcher	7	M	G-	-	-	-	+
98	Brin	Collie X	6	M	G-	-	-	-	+
99	Brecon	Sp Span	6	FN	G-	-	+	-	+
100	Frankie	Dob.	9	FN	G-	-	-	-	+
101	Bengie	Collie X	8	FN	G-	-	+	-	+
102	Tess	Collie X	3	FN	G-	-	+	-	+

Table 1f. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
103	Butch	JRX	10	M	G-	-	+	-	+
104	Sparky	BeagleX	6	MN	G-	-	+	-	+
105	Steffie	Lab.	7	FN	G-	-	+	-	+
106	Cassidy	JRX	8	M	G-	-	+	-	+
107	130595	Collie X	7	M	G-	-	+	-	+
108	Smudge	Collie X	8	FN	G-	-	+	-	+
109	129528	Dob.	7	M	G-	-	+	-	+
110	130650	CKCS	2	F	G-	-	-	-	+
111	130660	Ger. P.	4.5	M	G-	-	-	+	+
112	130686	Collie X	2	M	G-	-	+	-	+
113	130623	Sp Span	0.6	M	G-	-	+	-	+
114	130675	DH	2	F	G-	-	+	-	+
115	129709	Newf.	5	M	G-	-	-	-	+
116	129675	GSD	5	M	G-	-	+	-	+
117	130601	Cl. Span	6	M	G-	-	+	-	+
118	131992	Collie X	11	FN	G-	-	-	+	+
119	131910	GSD	6	F	G-	-	+	-	+

Table 1g. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
120	131737	Lurcher	6	M	G-	-	-	-	+
121	CDRM22	GSD	7	M	G+	-	+	-	-
122	CDRM23	GSD	8	M	G+	+	+	+	-
123	131870	GSD	10	M	G+	+	+	-	-
124	CDRM24	GSD	7	M	G+	-	+	-	-
125	133198	GSD	8	M	G+	-	+	-	-
126	CDRM25	GSD	10	M	R+	-	+	-	-
127	130884	GSD	10	M	G+	-	+	-	-
128	130417	GSD	11	M	G+	+	+	-	-
129	131167	GSD	7	M	G+	+	+	+	-
130	119643	G. Ret.	7	M	G-	-	+	-	-
131	Roz	GSDX	2	FN	G-	-	+	-	-
132	Iris	Sp. Span	2	FN	G-	-	+	-	-
133	GSD19	GSD	8	M	G-	-	+	-	-
134	GSD20	GSD	3	M	G-	-	+	-	-
135	Fergus	GSD	4.5	M	G-	-	+	-	-

Table 1h. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
136	GSD22	GSD	3	M	G-	-	+	-	-
137	GSD23	GSD	3	M	G-	-	+	-	-
138	Hamish	GSD	7.5	M	G-	-	+	-	-
139	Spot	GSD	4.5	F	G-	-	+	-	-
140	126400	GSD	7.5	M	G-	-	+	-	-
141	Fudge	Lab.	8	FN	G-	-	+	-	-
142	Jake	Collie X	9	MN	G-	-	+	-	-
143	120407	GSD	8	M	G+	+	-	-	-
144	124705	GSD	7	F	G+	+	-	+	-
145	129203	GSD	12	F	G+	+	-	-	-
146	CDRM1	GSD	10	M	G+	+	-	+	-
147	CDRM2	GSD	10	M	G+	+	-	+	-
148	127660	GSD	8	M	G+	+	-	-	-
149	129305	GSD	9	FN	G+	+	-	-	-
150	129415	GSD	12	F	G+	+	-	-	-
151	132623	GSD	10	M	G+	+	-	-	-
152	126583	GSD	9	M	G+	+	-	-	-

Table 1i. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
153	125737	GSD	10	M	G+	+	-	-	-
154	125157	GSD	9	F	G+	+	-	-	-
155	124365	GSD	8	M	G+	+	-	-	-
156	125462	GSD	8	M	G+	+	-	-	-
157	132146	GSD	10	FN	G+	+	-	-	-
158	125110	GSD	9	M	G+	+	-	-	-
159	125777	GSD	9	F	G+	+	-	-	-
160	124625	GSD	10	FN	G+	+	-	+	-
161	123913	GSD	13	M	G+	+	-	-	-
162	128040	GSD	12	M	G+	+	-	-	-
163	125570	GSD	9	MN	G+	+	-	+	-
164	126131	GSD	7	M	G+	+	-	+	-
165	127470	GSDX	9	FN	G+	+	-	-	-
166	126348	GSD	9	M	G+	+	-	-	-
167	127150	GSDX	10	MN	G+	+	-	+	-
168	125545	GSD	7	FN	G+	+	-	-	-
169	129664	GSD	7	FN	G-	-	-	+	-

Table 1j. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
170	125731	Lab.	7	M	G-	-	-	+	-
171	130383	A. Kar.	8	F	G-	-	-	+	-
172	130122	Gt Dane	0.58	F	G-	-	-	+	-
173	130401	Scot Ter	2	M	G-	-	-	+	-
174	129178	B. Terr.	6	M	G-	-	-	+	-
175	129950	Eng. P.	5	M	G-	-	-	+	-
176	130095	Lab.	9.5	M	G-	-	-	+	-
177	129428	B. Coll.	5	M	G-	-	-	+	-
178	129790	Gt Dane	0.66	F	G-	-	-	+	-
179	129454	DobX	7	FN	G-	-	-	+	-
180	129481	GSD	9	F	G+	-	-	+	-
181	129611	GSD	8	F	G-	-	+	-	-
182	127761	GSD	3	M	G-	-	-	+	-
183	128904	BMD	6.5	F	G-	-	-	+	-
184	129039	Dob.	9	M	G-	-	-	+	-

Table 1k. Signalment for all dogs included in this project. (For key see page 44).

A	B	C	D	E	F	G	H	I	J
No.	**Case ID**	**Breed**	**Age in years**	**Sex**	**CDRM status: G or R + or -**	**3**	**4**	**5**	**6**
185	129329	FCR	8	FN	G-	-	-	+	-
186	129365	Lab.	9	M	G-	-	-	+	-
187	116796	GSD	5.5	M	G-	-	-	+	-

Table 1l. Signalment for all dogs included in this project. CDRM status is given in column F as follows: "G" indicates diagnosis made at GUVS, "R" indicates diagnosis made in referral practice by Gary Clayton-Jones following further investigations as appropriate (*e.g.* survey radiography of the spine, myelography, EMG) "+" dog has CDRM and "-" dog has no evidence of CDRM. Columns G, H, I and J list the dogs included in results chapters 3, 4, 5 and 6 respectively. In column C the following abbreviations were used: German shepherd dog (GSD), Labrador (Lab.), Deer hound (DH), Jack Russell terrier (JR), Border collie (BC), Flat coat retriever (FCR), Springer spaniel (Sp Span), Doberman pinscher (Dob.), Cavalier King Charles spaniel (CKCS), German pointer (Ger. P.), Newfoundland (Newf.), Clumber spaniel (Cl. Span), Golden retriever (G. Ret.), Anatolian karabash (A. Kar.), Great Dane (Gt Dane), Bernese mountain dog (BMD), Scottish terrier (Scot. Ter). The suffix X denotes a crossbreed.

2.2.1 Ancillary investigations

In many cases the owners were unwilling to permit further investigations in order to rule out other differential diagnoses. In these cases, a typical history and clinical signs suggestive of CDRM in association with multiple re-examinations were necessary before the dogs were included in the study.

In several cases where it was thought likely that CDRM was not the problem, further diagnostic procedures were performed.

Routine biochemical and haematological evaluations were performed on thirty cases to investigate the possibility of systemic disease or to evaluate general health prior to anaesthesia. Plasma concentrations of urea, creatinine, sodium, potassium, chloride, calcium, phosphate, glucose, cholesterol, bilirubin, alkaline phosphatase, alanine transferase (ALT), aspartate transferase (AST), total protein, creatine kinase, albumin and globulin were analysed. Red blood cell count, haematocrit, haemoglobin concentration, mean corpuscular volume, mean cell haemoglobin, platelet count and total and differential white blood cell count were determined. Results of these tests were compared with reference ranges for University of Glasgow Veterinary School (GUVS) laboratories.

Radiographic, myelographic and electromyographic (EMG) examinations as well as cerebrospinal fluid (CSF) sampling were carried out under general inhalation anaesthesia. The precise anaesthetic regime depended on the procedure and individual patient requirements.

Survey radiography of the thoracolumbar spine and hips was performed as appropriate. Myelography was carried out in eight cases and involved injection of 0.3 ml/kg Iopamidol (Niopalm 300mg iodine/ml, Merck Pharmaceuticals) into the cisterna magna using the same protocol as described for CSF sampling. CSF samples were obtained from either the cerebellomedullary or lumbar cistern or both. The relevant collection site was clipped up and aseptically prepared. Cisterna magna samples were collected using a 21 gauge 1.5 inch hypodermic needle. The technique used is described by Evans [Method 1] (1995). Lumbar samples, where appropriate, were obtained *via* the L5/L6 interarcuate space as described by Lewis (1991) using a 20 guage 3.5 inch spinal needle (Monoject 220 Spinal Needle, Sherwood Medical Industries, St. Louis, U.S.A.). Samples were examined for colour, consistency and turbidity. The cell count and total protein concentration were determined and compared to reference values given by Bailey and Higgins (1985). In cases where the count exceeded five cells/μl in the cisterna magna or eight cells/μl in the lumbar cistern, a differential count was determined following

cytospin. A number of cases underwent electrophysiological examination using a Neuromatic 2000M electromyograph (Dantec Electronic, Denmark) and recordings of spontaneous and evoked muscle action potentials were made. Dogs were laid in lateral recumbency following general anaesthesia. EMG was performed using a concentric bipolar needle electrode as described by Bowen (1987). Pelvic limb muscles, in particular those demonstrating muscle atrophy or decreased muscle tone, were assessed for any abnormal electrical activity. EMG studies were carried out in seven cases.

From the history, clinical findings and lack of abnormalities found at further investigation, a tentative diagnosis of CDRM was made. Cases were monitored on a regular basis throughout the course of their disease and, with the owners' permission, a post mortem examination was performed following euthanasia.

2.2.2 Gait scoring system

A scoring system was devised to allow a more objective assessment of the progression of disease. This involved assessment of gait analysis at each examination.

Analysis of Gait

Gait Score	Gait Analysis
0	Normal
1	Occasionally scuff hind feet when circling or on stairs
2	Scuff dorsi (hind feet) at slow pace and on stairs. Cross pelvic limbs on circling.
3	Circling very difficult, may fall over.
4	Collapse to one side when moving in a straight line.
5	Loss of useful pelvic limb function, able to pull along with thoracic limbs.
6	Loss of useful pelvic limb and thoracic limb function

Table 2. Gait scoring analysis.

2.2.3 Collection of samples

Affected dogs were examined in the GUVS clinic every two to three months. At each visit a number of blood samples were collected. On the first visit to the clinic, routine haematology and biochemistry screens were carried out, in 30 of the dogs, to check for any intercurrent diseases. These were processed by the relevant laboratories at GUVS and compared with normal values for these laboratories. A 20ml jugular sample was collected; five ml was collected into a plain tube, and allowed to clot at room temperature for 30 to 60 minutes before undergoing a 1400g spin in a Beckman GP centrifuge for 15 minutes at room temperature. The serum was carefully removed using a one ml pipetteman and divided and stored in 1.5 ml eppendorf vials at -70°C. This was used to measure serum vitamin E levels, see 2.2.3.1 Measurement of serum vitamin E (page 47).

A further 10ml was collected into EDTA(K_2), and stored at either 4°C for genomic DNA (gDNA) extraction within five days, otherwise at -20°C. The procedure used for routine gDNA extraction was influenced by the potential downstream application of Southern analysis and the Repeat Expansion Detection (RED) technique, with their requirements for reproducibility and high quality. A number of commercially available kits were assessed. These kits are marketed primarily as offering high quality gDNA from procedures with reduced manipulations (saving on time) and increased safety by removing the need for phenol-chloroform extraction. The various kits selected were based on different principles. In all cases contaminating RNA was removed with RNAse A treatment (see 8.5.1 RNase A, page 215) the details of which varied between protocols. The Nucleon II kit (Scotlab) was chosen as being the most suitable for the purposes of this project.

2.2.3.1 Measurement of serum vitamin E

Samples were sent to the Scottish Agricultural College (SAC) at Auchincruive where the serum vitamin E levels were measured using the serum α-tocopherol High Performance Liquid Chromatography (HPLC) method. Vitamin E standard (Kodak Chemical Co. Liverpool) 2μg/ml in 0.5ml ethanol and internal standard (100μl of 2.5μg/ml tocol in ethanol) were diluted with 0.5ml distilled water. 0.5ml serum samples and internal standard (as above) were precipitated with 0.5ml ethanol. The vitamin E was extracted from samples and standards into 2.5ml hexane by vigorous mixing followed by centrifugation at 1500rpm for 5 minutes. As much as possible of the hexane layer was taken off and dried on a Dri-block heater under oxygen free nitrogen (BOC gases) at 50°C and then redissolved in 300μl methanol (BDH) before transfer to autosampler vials for vitamin E determination. Separation of the end products was achieved by HPLC, using a

15cm by 4mm i.d. Superspher 4μm RP18 column with a Lichrocart 4-4 guard column. Degassed methanol:water 98:2 was used as eluent, flowing at 1.5ml/min from a Spectra Physics SP8770 isocratic pump. A Gilson 232 Bio autosampler was used to inject 110μl through the 20μl loop the contents of which were then flushed onto the column. Detection was achieved using a Jasco 821-FP fluorimeter with 296nm excitation and 330nm emission wavelengths. Results were collected and integrated using a Spectra Physics Chromjet integrator. Retention times for tocol and vitamin E were about three and five minutes respectively. The concentration of α-tocopherol is calculated by comparison to an α-tocopherol standard curve (using peak areas).

2.3 TISSUE FIXATION AND PROCESSING

2.4 Fixation

Preparation of all fixatives, buffers, stains *etc.* are given in 8 Appendix (page 201), specific page numbers being given in the heading.

2.5 Fixatives

2.5.1 Buffered neutral formaldehyde, 4% (BNF) (page 202)

This was used for the preservation of tissues used for embedding in paraffin wax.

2.5.2 Karnovsky's modified fixative (paraformaldehyde/glutaraldehyde 4%/5%) (page 202)

This was used for the preservation of tissue destined for embedding in Araldite resin.

2.6 Techniques

Brain and spinal cord were removed by the routine procedure used in the post mortem room at GUVS. During removal of the spinal cord both the dorsal and ventral aspects of the vertebral canal were examined for the presence of lesions which could potentially have caused spinal cord compression.

2.6.1 Immersion

Brain, spinal cord and body tissues were immersion-fixed in 4% BNF for a minimum of 24 hours. Spinal cords were suspended and weighted to prevent curling during fixation. Brains were cut transversely at the level of the optic chiasma to ensure adequate fixation throughout the tissue. The following body tissues were collected from ten of the CDRM cases post mortemed; stomach, duodenum, jejunum, caecum, ileum, colon, thyroid gland, adrenal gland, pancreas, kidney, liver, spleen as well as optic nerve, sciatic nerve, stellate ganglion and cranial mesenteric ganglion.

2.6.2 Intra-aortic perfusion

Tissues required for electron microscopy were perfusion-fixed in Karnovsky's modified fixative. All perfusions were carried out in a suitably designated room, the solutions were administered by gravity. Euthanasia was carried out by intravenous injection with 20ml of pentobarbitone sodium (Euthatal) (Rhone Merieux Ltd.). This was followed immediately by 10ml of heparin (Heparin injection BP) (Leo Laboratories Ltd.) to prevent clotting. The dog was lifted onto the table in right lateral recumbency. The left chest wall was removed to allow access to the dorsal aorta. A small incision was made into the aorta and a rubber tube inserted and tied in position. A cut was made in the right auricle to allow the blood to escape. The vascular system was initially flushed with five litres of 0.85% saline, followed by eight litres of Karnovsky's modified fixative. The flow rate for these fluids was 13 litres per hour. The head of pressure was two metres.

2.7 Processing

2.7.1 Paraffin processing

Tissues for paraffin wax embedding were processed using one of two Shandon Elliot automatic tissue processors (Histokinettes). Spinal cord and body tissues were processed in the 24 hour histokinette while brain was processed in the seven day histokinette. For processing protocol see page 204.

All tissues were subsequently embedded in paraffin wax (Merck) at 60°C.

2.7.2 Resin processing

Samples were processed using a Lynx *el* microscopy tissue processor (Leica) for embedding in Araldite resin (page 205).

2.7.3 Sections

2.7.3.1 Paraffin-embedded tissue

Paraffin-embedded tissue sections were cut on a Biocut 2035 microtome (Leica) at eight μm for routine histology and six μm for immunocytochemistry, mounted onto APES-coated microscope slides and allowed to dry overnight in a 60°C oven.

2.7.3.2 Preparation of APES coated slides

Cleaned slides were coated with 3-aminopropyltriethoxy-silane (APES) (Sigma). Slides were washed overnight in the detergent Decon 90 (5%) (Decon Lab Ltd) to remove grease, washed well in distilled water (DW) and oven dried wrapped in foil. Slides were soaked, in a fumehood, in 0.25% APES in methylated spirits for 2mins followed by 0.01% DEPC SDW for 2mins prior to oven drying wrapped in foil. Slides were stored at room temperature.

2.7.3.3 Resin sections

Sections for light microscopy were cut at one μm on an Ultracut E ultratome (Reichert-Jung) and mounted on sulphuric acid-washed slides.

2.7.3.4 Ultra-thin sections

Sections for electron microscopy were cut at approximately 70nm (ultratome as above) and mounted on 200 mesh-3.06mm diameter copper grids.

2.7.4 Staining Techniques

2.7.4.1 Light microscopy

2.7.4.1.1 Routine stains

2.7.4.1.1.1 Haematoxylin and eosin (H&E)

Sections from paraffin blocks were stained routinely with H&E for routine assessment of tissue morphology. Details of the technique are given on page 206. Sections were mounted in DPX mountant (BDH).

2.7.4.1.1.2 Haematoxylin

A number of immunocytochemistry sections were counterstained using haematoxylin to identify cell nuclei. Details of the technique are given on page 207. Sections were then dehydrated and mounted as for H&E staining.

2.7.4.1.1.3 Cresyl violet (CV)

Brain and spinal cord sections from paraffin blocks were stained with cresyl violet to demonstrate Nissl substance. Details of the technique are given on page 207. Sections were dehydrated and mounted as for H&E staining.

2.7.4.1.1.4 Congo Red

Sections of red nucleus from paraffin blocks were stained with Congo Red to investigate whether or not amyloid was present. Details of the technique are given on page 208. Sections were dehydrated and mounted as for H&E staining.

2.7.4.1.1.5 Methylene blue/azur II

Resin sections were stained routinely with methylene blue/azur II (see page 210) to demonstrate myelin. Slides were heated on a hot plate to 60°C, flooded with stain for 10-30 seconds then rinsed in running tap water. They were dried overnight on the hot plate and mounted in DPX (BDH).

2.7.4.1.2 Immunocytochemistry

2.7.4.1.3 Peroxidase anti-peroxidase (PAP) technique

The peroxidase anti-peroxidase technique was carried out on six μm paraffin-embedded sections. The primary antibodies, sources, dilutions and links are summarised in Table 3, page 54. Sections were initially hydrated, endogenous peroxidase activity was quenched by immersing slides in 3% hydrogen peroxidase (in absolute alcohol) for 30 minutes followed by washing in running water for 30 minutes. Non-specific binding was blocked by incubation in 10% normal goat serum (NGS) in PBS for two hours at room temperature. Sections were incubated in the primary antibody in 1% NGS in PBS overnight in a humidifying chamber at 4°C.

Sections were adjusted to room temperature then washed for 30 minutes in six changes of PBS. The secondary antibody (link) in 1% NGS in PBS at room temperature was applied to the sections and left one hour. Excess antibody was removed by washing in PBS for 30 minutes (as before) before incubating sections in the PAP complex diluted in 1% NGS in PBS, for 30 minutes at room temperature. Again, excess was removed by serial washes in PBS over 30 minutes. The chromogen was developed in filtered 0.1M phosphate buffer (pH 7.3) containing 0.5mg/ml 3,4,3`,4`,-tetraminobiphenyl hydrochloride (DAB) and 0.003% hydrogen peroxide until the required colour intensity had been achieved (up to five minutes).

Sections were washed in running water, or counterstained in haematoxylin, dehydrated and mounted in DPX.

2.7.4.1.4 ABC technique

The ABC technique bore some resemblance to the PAP technique but differed in that the secondary antibody was biotinylated and the colour reaction used a preformed avidin and biotinylated horseradish peroxidase macromolecular complex called Vectastain (Vectastain *Elite* ABC Kit, Vector Laboratories). The primary antibodies, sources, target antigens, and range of dilutions are summarised in Table 4, page 55.

Paraffin-embedded sections were initially dewaxed in histoclear and hydrated by passage through graded alcohols before being rinsed for five minutes in water. Quenching of endogenous peroxidase activity involved incubation in 3% hydrogen peroxide in ethanol at room temperature followed by washing in PBS for 20 minutes. Sections were incubated for two hours with 10% NGS blocking serum. Excess serum was removed by blotting. Sections were incubated overnight at 4°C with the primary antiserum diluted in 1% NGS in PBS. Sections were allowed to reach room temperature and washed in PBS for 10 minutes.

Sections were incubated for 30 minutes with diluted biotinylated secondary antibody solution before being washed for 10 minutes in PBS. The chromogen was applied *i.e.* Vectastain Elite ABC reagent (for details see page 209) and sections incubated for 30 minutes. Excess was then removed by a 10 minute wash in PBS. Sections were incubated in peroxidase substrate solution until the desired staining intensity had developed.

Negative controls used non-immune mouse and rabbit sera, as appropriate, *in lieu* of primary antibodies.

2.7.4.1.5 Immunofluorescence

Immunofluorescence double staining was carried out on paraffin-embedded sections of spinal cord. The primary antibodies, dilutions, sources and secondary antibodies used are summarised in Table 5, page 56. Sections were initially dewaxed and hydrated, washed briefly in PBS before incubation with the two primary antibodies (these were made up at double strength in 1% NGS in PBS, then added simultaneously) overnight at 4°C in a humidifying chamber. Sections were adjusted to room temperature and washed three times in PBS.

Detection involved addition of the relevant fluorochrome-labelled secondary antibodies (made up at double strength in PBS then both added together as before) which were left at room temperature for 30 minutes. Sections were washed in water for 20 minutes before being wet-mounted in Citifluor antifade mountant (page 209). Sections were stored at 4°C in a lightproof box if immediate photography was not possible.

2.7.4.2 Electron microscopy

Blocks of tissue were processed for embedding in Araldite resin (see 2.7.2 Resin processing, page 49). Sections for light microscopy were cut at one μm on a Reichert-Jung Ultracut E ultratome, then stained with methylene blue/azur II (page 210). Thin sections for electron microscopy were cut at 70nm and placed on 200 mesh copper grids. Grids were stained with uranyl acetate and lead citrate (page 210).

Primary Antibody	Target antigen	Range of dilutions	Source	Link	PAP Complex	Source
SMI 31 (mouse monoclonal)	phosphorylated neurofilaments	1/3000-1/5000	Affiniti	Goat anti-mouse (1/10)	mouse (1/1250)	Sigma
RT-97 (mouse monoclonal)	phosphorylated nerofilaments	1/1000-1/1500	Drs. Wood & Anderton, London	Goat anti-mouse (1/10)	mouse (1/1250)	Sigma
RM017 (mouse monoclonal)	phosphorylated neurofilaments	neat	Dr V. M-Y. Lee, Philadelphia	Goat anti-mouse (1/10)	mouse (1/1250)	Sigma
RM024 (mouse monoclonal)	phosphorylated neurofilaments	neat	Dr V. M-Y. Lee, Philadelphia	Goat anti-mouse (1/10)	mouse (1/1250)	Sigma
α-GFAP (rabbit polyclonal)	astrocytes	1/4000-1/5000	Dako	Goat anti-rabbit (1/10)	rabbit (1/40)	ICN immuno biologicals
α-PLP 226 (rabbit polyclonal)	C-terminal of PLP/DM20	1/600	Prof. N. P. Groome, Oxford	Goat anti-rabbit (1/10)	rabbit (1/40)	ICN immuno biologicals
NA 1297 (rabbit polyclonal)	neurofilament proteins of varying sizes	1/1000-1/1500	Affiniti	Goat anti-rabbit (1/10)	rabbit (1/40)	ICN immuno biologicals
α-ubiquitin (rabbit polyclonal)	ubiquitin	1/300-1/500	Dako	Goat anti-rabbit (1/10)	rabbit (1/40)	ICN immuno biologicals
α-MBP (rabbit polyclonal)	myelin basic protein	1/400	Dr J. M. Mattieu, Lausanne	Goat anti-rabbit (1/10)	rabbit (1/40)	ICN immuno biologicals

Table 3. Antibodies, target antigens, dilutions, link and sources for immunostaining by the PAP technique.

Primary Antibody	Target antigen /tissue	Range of dilutions	Source	Biotinylated Link	Biotinylated Link
α-MAP 5 (mouse monoclonal)	Purkinje cells	1/2000	Sigma	Horse anti-mouse IgG 1/100	Horse anti-mouse 1/100
α-Calbindin-D (mouse monoclonal)	Purkinje cells	1/200	Sigma	Horse anti-mouse IgG 1/100	Goat anti-rabbit 1/100
α-MAP 2 (mouse monoclona)l	Purkinje cells	1/750-1/1000	Sigma	Horse anti-mouse IgG 1/100	Goat anti-rabbit 1/100
TP 70 (rabbit polyclonal)	C-terminal tau	1/5000	Prof. B.H. Anderton, London	Goat anti-rabbit IgG 1/100	Horse anti-mouse 1/100
TP 007 (rabbit polyclonal)	N-terminal tau	1/4000	Prof. B.H. Anderton, London	Goat anti-rabbit IgG 1/100	

Table 4. Primary antibodies, target antigens, range of dilutions and sources for immunostaining by the ABC technique.

Primary Antibodies	Final Dilutions	Source	Secondary Antibodies	Dilution	Source
SMI-31	1/1500	Affiniti	Goat anti-mouse IgG_1	1/75	S. Biotech
GFAP	1/750	Dako	Biotinylated Horse α-Rabbit IgG + Extra avidin FITC	1/75	Vector Laboratories
PLP 226	1/600	Prof. N.P.Groome Oxford	Goat anti-mouse IgG_1	1/75	S. Biotech
SMI 31	1/1500	Affiniti	Biotinylated Horse α-Rabbit IgG + Extra avidin FITC	1/75	Vector Laboratories

Table 5. Antibodies, dilutions, sources and secondary antibodies used in immunofluorescence.

2.8 Molecular Genetics Techniques

All solutions were prepared and sterilised as appropriate. Bulk solutions were autoclaved while small volumes and fluids which were unsuitable for autoclaving were filter sterilised using a Flowpore 0.45μm filter (Biomedicals Ltd.).

2.8.1 Genomic DNA (gDNA) extraction

2.8.1.1 gDNA extraction from blood

Blood was collected into 5ml EDTA(K_2) tubes as detailed previously. Subsequent storage was at 4°C if the DNA was to be extracted within five days of collection, otherwise at -20°C. The Nucleon II Kit (Scotlab) was used to extract gDNA from blood. This technique was based on the principle of separating DNA from cell impurities using the proprietary resin Nucleon®. Expected yield from five ml of blood was 150-220μg gDNA.

Blood samples at room temperature were transferred into sterile 40ml polypropylene centrifuge tubes. Nucleon II cell lysis solution, 30ml, (details on page 215) was added to each. The tubes were rotated at room temperature for four minutes then spun in a Beckman J2-21M/E centrifuge for four minutes at 4000rpm. The supernatant was poured off carefully leaving the cell pellet. Two ml of Nucleon II washing solution (for details see page 215) was added to the cell pellet and vortexed to resuspend the cells. The suspension was transferred to a 15ml chloroform-resistant Falcon tube, 15μl of a 50μg/ml RNase A solution was added and incubated at 37°C for 30 minutes. Deproteinisation involved the addition of sodium perchlorate to a final concentration of 1.25M, a 15 minute rotary mix at room temperature, followed by a 25 minute incubation in a 65°C shaking water bath.

Two ml of cold chloroform was added to each tube. The tubes were rotary-mixed for 10 minutes before centrifugation at 857g (Labofuge 400, Heraeus) for one minute. Without disturbing the separate layers, 300μl of Nucleon silica suspension were added to each tube before a further spin, at 1400g, was carried out for three minutes. At this stage any gDNA is above the silica suspension layer. This top layer was transferred into a fresh Falcon tube. Some of the silica was inevitably transferred, this was removed by a four minute spin at 1300g, and transferred to a fresh tube. Two equal volumes of cold absolute alcohol were added to the aqueous phase. Gentle inversion of the tubes resulted in precipitation of the gDNA. gDNA pelleted by spinning for 5 minutes at 4000g was washed in 70% alcohol. The gDNA was allowed to air dry before resuspension in an appropriate volume of TE buffer, pH 8.0.

2.8.1.2 gDNA extraction from tissues

Tissues were snap frozen and stored in liquid nitrogen.

a) small scale gDNA extraction from tissues

gDNA was extracted using the QIAamp® Tissue Kit (Qiagen), which involved using an ion exchange resin which selectively binds gDNA. gDNA can be washed from the column and concentrated by ethanol precipitation. An RNase A step was included. The yield of gDNA was dependent on the tissue type used, 25mg of brain or liver should yield 15 to 30 μg gDNA; 10mg of spleen should yield five to 30μg gDNA.

b) large scale gDNA extraction from brain

This technique was applied for the extraction of gDNA from 200 mg to one g samples of brain. The tissue was weighed, then crushed in a mortar and pestle in the presence of liquid nitrogen. The powdered tissue was suspended in 1.2ml of digestion buffer (for details see page 215). This mixture was incubated at 50°C for 12 to 18 hours. Once the samples were viscous, any RNA was removed by the addition of 0.1% SDS and one μg/ml DNase-free RNase A (for details see page 215) and incubation at 37°C for one hour. Deproteinisation of nucleic acids was performed using phenol:chloroform:isoamyl alcohol (25:24;1) (Sigma) extraction. An equal volume of phenol:chloroform was added to the nucleic acid sample in a polypropylene tube with a tight fitting lid. The contents were mixed until an emulsion formed, this was centrifuged at 12000g for 15 minutes at room temperature. The phases separated and the aqueous phase containing the gDNA was transferred to a clean tube. This organic extraction step was repeated until there was no longer a white precipitate at the interface. Purification of the gDNA was carried out by ethanol precipitation. gDNA was precipitated routinely in the presence of 0.3M sodium acetate ($NaAc^-$), pH 5.2, and three volumes of ice cold ethanol. Amounts less than one μg were precipitated overnight at -70°C. Larger amounts were precipitated either at -70°C for at least an hour, or -20°C for at least two hours. Samples were centrifuged at 4°C for 15 to 30 minutes at 13000rpm and the supernatant removed. The gDNA pellet was washed with 70% ethanol and centrifuged at 4°C for 5 minutes at 13000rpm. The pellet was air dried for up to 10 minutes before reconstitution in the appropriate volume of TE buffer (for details see page 211). Expected yield from this technique was approximately two mg DNA per one g of tissue.

2.8.2 Genomic DNA assessment

2.8.2.1 Quantity

gDNA samples were quantitatively assessed by spectrophotometry (Genequant Spectrophotometer, Pharmacia Biotech). Working concentrations were a mean of two readings, of different dilutions, the results of which had to be within 10% of each other. The concentrations of small quantities of DNA were estimated by gel analysis, against varying aliquots of an appropriate sample of known concentration.

2.8.2.2 Quality

The integrity of the gDNA was assessed qualitatively by electrophoresis on a 0.8% agarose mini-gel (for details see 2.8.3 Nucleic acid electrophoresis, page 59). This was followed by a check on the digestibility of the gDNA. One µg samples were set up in three conditions, one digestion at 37°C in the presence of 30 units of a six-cutter enzyme for two hours, a second incubated at room temperature for the same length of time and a third incubated at 37°C without the enzyme (Figure 2, page 60).

2.8.3 Nucleic acid electrophoresis

2.8.3.1 Agarose gels

Routine electrophoresis of DNA was performed using 0.7 to 2% regular agarose gels of ultra-pure electrophoresis-grade agarose (Gibco BRL). Gels were cast by melting the agarose in the presence of Tris acetate EDTA buffer (TAE) (0.04M Tris acetate, 0.001M EDTA), and were run in the same buffer. The gel was cooled to 60°C and ethidium bromide was added to a final concentration of 0.5µg/ml. Samples were loaded with a suitable amount of a 6x gel loading buffer (1x TAE; 30% glycerol (Sigma); 0.25% bromophenol blue (BDH Chemicals Ltd.); 0.25% xylene cyanol FF (Sigma). Gels were viewed using a "Fotoprep I" ultraviolet transilluminator (Fotodyne Inc.) and photographed using a Polaroid MP4 land camera (Polaroid), a T2201 transilluminator (Sigma), a Wratten 22A filter (Kodak) and Polaroid 665 (ASA 80) film.

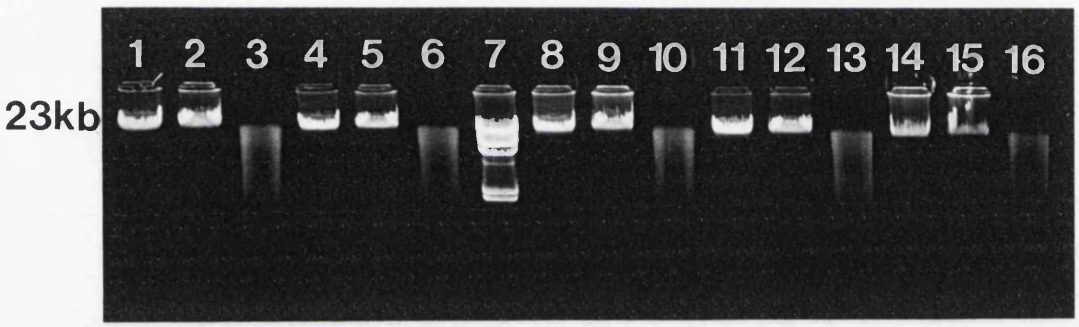


Figure 2. Assessment of the quality of gDNA extracted from fresh (lanes 1-6 and 8-13) and frozen (lanes 14-16) canine blood samples. Lanes 1, 4, 8, 11, 14 gDNA incubated at room temperature; lanes 2, 5, 9, 12, 15 incubated at 37°C in the presence of buffer and the absence of enzyme; lanes 3, 6, 10, 13, 16 *Eco* RI digest at 37°C; lane 7 λ *Hin*d III marker.

2.8.4 Amplification of specific genomic regions harbouring the CAG repeats by PCR

Primer pairs were obtained from Cruachem (Table 6, page 61).

PCR Product	Forward primer	Reverse primer	Product length (bp)
ataxin-1	5/CAACATGGGCAGTC TAGAG 3/	5/AACTGGAAATGTGG ACGT 3/	214
atrophin-1	5/CACCAGTCTCAACA CATCACCATC 3/	5/CCTCCAGTGGGTGG GGAAATGCTC 3/	140

Table 6. PCR primer pairs for amplification of specific genomic regions that have been demonstrated to harbour CAG repeats in man (See 6.2.2 Neurodegenerative conditions resulting from CAG repeat expansions, page 164).

All amplifications were carried out on a Perkin Elmer DNA thermal cycler. Reactions were carried out in 50µl volumes in 0.5µl eppendorfs overlaid with 50µl of molecular biology grade mineral oil (Sigma), to prevent evaporation. Reactions contained 100ng of target gDNA, 0.2mM dNTP's, 0.3µM of the forward and reverse primers, 1.5 to 5mM magnesium chloride as appropriate, 2% formamide if necessary, and in the presence of 2.5U Taq polymerase (Bioline) and ammonium buffer (Bioline) (20mM Tris HCl pH 8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% Glycerol, 0.5% Tween-20). Thirty nine cycles were used for all primer pairs. An initial denaturation step at 94°C for one minute 30 seconds was followed by 38 cycles of annealing at 60°C (dependent on primer pair in use) for 45 seconds, extension at 72°C for one minute and denaturation at 94°C for 45 seconds. The final cycle consisted of an annealing step at 55°C for one minute and an extended extension step at 72°C for five minutes, followed by a 4°C soak cycle.

2.8.5 Southern blotting

2.8.5.1 DNA radiolabelling

Random primed ^{32}P labelled DNA probes were generated from the DM 200 cloned PCR product which was comprised of ~100 base pairs of human flanking DNA and 200 CTG repeats from the human Myotonic Dystrophy locus (DM) using the "High prime" labelling kit (Boehringer Mannheim). This was an optimised mixture of random hexamers, dNTPs and Klenow enzyme in 5x buffer containing 0.5M Tris

HCl (pH 7.5), 0.1M $MgCl_2$, and glycerol. The kit was optimised for use with $^{32}P\alpha$ dCTP.

30ng of fragment in a final volume of 11µl of SDW was denatured in a boiling water bath for 10 minutes then quenched on ice. This was used as template in a 20µl reaction containing four µl "High Prime" solution and five µl (50µCi) $^{32}P\alpha$ dCTP, 3000Ci/mMol, aqueous solution. These were mixed thoroughly and incubated at 37°C for 10 minutes. The reaction was stopped in the presence of 10mM EDTA and incubated at 65°C for 10 minutes.

2.8.5.2 Removal of un-incorporated nucleotides

Un-incorporated nucleotides were removed by passage of the labelled mixture through a Nick column (Pharmacia Biotech) which contain Sephadex® G-50 beads and allowed rapid purification of labelled DNA (20 base pairs and larger) from unincorporated radiolabelled nucleotides by size exclusion chromatography. The eluted labelled DNA fragment was stored at -20°C until use. Routine specific activities were 7-9x 10^8 cpm/µg DNA.

2.8.5.3 Restriction enzyme digestions

Restriction enzymes were supplied by Gibco BRL, Promega and New England Biolabs and used according to manufacturers' instructions. The following restriction enzymes were used in combination:

Digest	Restriction Enzymes
1)	*Hind* III + *Bam* HI
2)	*Hae* III + *Hha* I
3)	*Hae* III + *Hha* I + *Sau* 3AI
4)	*Bam* HI + *Hind* III + *Hae* III + *Hha* I + *Sau* 3AI

Table 7. Restriction enzymes used in creating restriction fragment profiles.

2.8.5.4 Gel preparation

Digested DNA was run on agarose gels of 1.25 to 2% concentration, using a mid-gel system (Model H3-SET, Anachem) with a bed volume of 15 x 13 cms. Two DNA size markers were used as appropriate, λ *Hind* III and 100 base pair DNA ladder. A photographic record was kept to aid interpretation of the autoradiograph. The size-

separated gDNA fragments were denatured by soaking the gel in denaturing solution (0.5M NaOH/1.5M NaCl) in a covered plastic dish and shaking gently for 30 minutes on a platform shaker. The gel was washed twice in distilled water, rinsed and shaken in neutralisation solution (1.5M NaCl/0.5M Tris pH 7.4) for 45 minutes, twice, in order to lower the pH to below 9.0.

2.8.5.5 Transfer

DNA was transferred using a Model 785 vacuum blotter, liquid trap, vacuum regulator and pump (Biorad) for 90 minutes in 10xSSC with a pressure of five in. Hg onto a nylon filter (Hybond-N, Amersham). After transfer, filters were washed in 2xSSC to remove any gel fragments before blotting dry on Whatman® filter paper. Nucleic acids were immobilised by UV crosslinking using an XLL 100 (Spectronics Corporation).

2.8.5.6 Prehybridisation

Prehybridisation was carried out in a hybridisation oven rotisserie (Micro-4, Hybaid) using Rapidhyb buffer (Amersham). The volume of Rapidhyb depended on the size of bottle used, and varied from five ml for the small bottle, to 15ml for the large bottle. The oven, bottles and hybridisation buffer were preheated to 65°C. Prehybridisation was carried out for a minimum of two hours.

2.8.5.7 Hybridisation

Labelled probe was denatured for 10 mins at 95°C. The reaction was quenched on ice. Approximately two ng of heat-denatured labelled probe was added per one ml of Rapidhyb solution (Amersham). Initially, a one ml aliquot of the prehybridisation solution was removed from each bottle and the labelled probe added. This was returned to the bottle, ensuring even mixing of the probe. Hybridisation was carried out overnight at 65°C.

2.8.5.8 Washing

Washes were carried out in a shaking water bath at 65°C. The first two washes were carried out in 2xSSC, 0.1% (w/v) SDS for 30 minutes. The third and fourth washes were carried out in 0.1xSSC, 0.1% (w/v) SDS, for 30 minutes each wash. Reduction in background levels of probe was monitored using a mini-monitor, the stringency and length of washes were then altered as necessary.

2.8.5.9 Autoradiography

Hybridised filters were allowed to air dry and placed in heat-sealed plastic bags. Autoradiography was performed using Cronex 10S X-ray film (Dupont) with Kodak "X-Omatic" regular intensifying screens for the appropriate times.

2.8.6 Repeat Expansion Detection (RED) technique

2.8.6.1 DNA radiolabelling

$^{32}P\alpha$ dCTP Redivue (Amersham) was used to radiolabel a $(CAG)_{10}$ using terminal deoxynucleotidyl transferase which, in the presence of a divalent cation, catalysed the addition of dNTPs to the 3/-hydroxyl termini of DNA molecules. The reaction mixture contained one µg $(CAG)_{10}$, 50µCi $^{32}P\alpha$ dCTP, 15U TdT (GibcoBRL), five µl TdT buffer (x10) which contained 0.5M potassium cacodylate (pH 7.2), 10mM $CoCl_2$ and one mM dithiothrietol (DTT) made up to 50µl with SDW. This was incubated at 37°C for one hour. The reaction was placed on ice and quenched by the addition of EDTA to a final concentration of 10mM. Ethanol precipitation (see page 2.8.1 Genomic DNA (gDNA) extraction, page 57) was carried out with one adaptation, one µl of glycogen at 20mg/ml was added to the reaction mixture to ensure pellet visibility. Specific activity levels of >1.4 x 10^7cpm/µg DNA were obtained.

2.8.6.2 Kinase reaction

Suitable quantities of fresh $(CTG)_{17}$ were phosphorylated immediately prior to each ligation. This involved the addition of 50ng $(CTG)_{17}$, three U T_4 polynucleotide kinase (PNK) (New England Biolabs) in the presence of 50mM dATP, 10xPNK buffer (70mM Tris HCl, 10mM $MgCl_2$, 5mM DTT pH 7.6), were adjusted to five µl with SDW for each downstream reaction. The mixture was incubated at 37°C for one hour.

2.8.6.3 Ligation

The ligation reaction was carried out in two steps. Ten µg of target gDNA was added to 500ng of the phosphorylated CTG_{17} and 2µl SDW to give a reaction volume of 17µl in a 0.5ml eppendorf. This reaction mixture was incubated for 5 minutes at 95°C before the addition of 5U Ampligase (Epicentre Taq Ligase, UK supplier; Cambio) and 2µl of 10x Ampligase buffer containing 50mM Tris-HCl (pH 7.5), 100mM NaCl, 0.1mM EDTA, 0.1% Triton® X-100 and 1mM dithiothreitol (DTT). Reagents were then overlaid with 20µl mineral oil (Sigma).

Positive internal controls were supplied by using mouse gDNA (Jackson Labs., Maine). The strain used was 690 129J which had been cut with *Hind* III and was known to result in a suitable ladder.

2.8.6.4 Thermocycling conditions

The annealing and ligation steps were carried out in a Perkin Elmer DNA Thermal cycler. An initial denaturation of the phosphorylated $(CTG)_{17}$ and target DNA was carried out at 94°C for five minutes before the addition of the Ampligase and Ampligase 10x buffer. This was followed by 396 cycles of a two-temperature cycling protocol with ten seconds at 94°C followed by 30 seconds at 70°C. The Ampligase enzyme and buffer were replenished after 99 cycles.

The product was extracted from under the mineral oil by direct removal of the lower aqueous layer.

2.8.6.5 Gel preparation

Resolution of ligation products was achieved by preparing a 6% denaturing polyacrylamide gel containing 6M urea in a buffer of 100mM Tris borate, (pH 8) one mM EDTA for two hours 30 minutes at 1500V constant voltage at 50°C. This was performed on a sequencing gel rig (Model V4-II, Anachem). A gel measuring 15cm by 40cm and 0.4mm thick was prepared as follows; preprepared 40% bis-acrylamide was stored at 4°C (for details see page 8.6.2 Polyacrylamide gel compositions, page 217); to 15ml of this was added 42g of urea and 20mls of 5xTBE buffer; SDW was added to a final volume of 100mls. The urea was solubilised by warming. The solution was allowed to cool before the addition of 35µl of N,N,N`,N`,-tetramethylethylenediamine (TEMED) and 400µl of 10% ammonium persulphate (APS), immediately prior to pouring the gel. Following removal of the comb the wells were flushed to rid them of urea.

2.8.6.6 Transfer

The gel was transferred onto a Hybond N+ membrane (Amersham) by capillary transfer on the bench overnight. The membrane was wet in distilled water and equilibrated by soaking in 1xTBE buffer for ten minutes. Five pieces of Whatman® 3MM paper were cut to the size of the gel. On completion of the gel run, the gel rig was laid flat on the bench and one of the glass plates carefully removed. The gel was rinsed with 1xTBE, which was then rolled off with a plastic pipette. The soaked membrane was carefully placed over the gel, rolling again to exclude any bubbles. One sheet of 3MM was wet in distilled water and laid over the filter, the other four (dry) 3MMs were also placed on top, the second glass plate was replaced.

Following transfer for five hours the filter was removed from the sandwich and soaked in 5xSSC for up to one minute to ensure no fragments of polyacrylamide were still attached. The membrane was left on filter paper for up to one hour to dry then UV-crosslinked (XLL 100, Spectronics Corporation).

2.8.6.7 Prehybridisation

Prehybridisation was undertaken in a hybridisation oven with rotisserie (Micro-4, Hybaid) using 15ml of Rapidhyb hybridisation buffer (Amersham). Oven, bottles and solutions were all preheated to 65°C. Prehybridisation was carried out for a minimum of five hours.

2.8.6.8 Hybridisation

Approximately 100ng/ml of labelled probe was added to the prehybridisation solution. A small volume of the prehybridisation solution was transferred into a universal, mixed with the labelled probe, and poured over the filter. Hybridisation was carried out overnight at 65°C.

2.8.6.9 Washing

The filters were initially washed at 65°C in 5xSSC, 0.1% (w/v) SDS for ten minutes. The waterbath temperature was then altered to 42°C and a second wash was carried out in 0.1xSSC, 0.1% (w/v) SDS for ten minutes. The reduction in background levels of probe was determined using a mini-monitor, allowing the stringency and length of washes to be changed as necessary.

2.8.6.10 Autoradiography

Hybridised filters were allowed to air dry, then placed in heat-sealed plastic bags. Autoradiography was carried out using Kodak X-Omat AR X-ray film with Kodak "X-Omatic" regular intensifying screens for four days and 24 days at -70°C.

2.8.7 Western blot analysis

2.8.7.1 Sample preparation

Protein was isolated from 200mg samples of canine brain by homogenisation in 0.1M Tris (pH 6.8), 1mM Nα-p-tosyl-l-lysine chloro-methyl ketone (TLCK), 2% SDS and boiled for 5 minutes. Samples were centrifuged at 13000g for five minutes in a Heraeus Biofuge at 4°C, the supernatant was retained and stored at -20°C. Total protein was measured using the BCA protein assay reagent (Pierce) (see page 8.6.1 Pierce Protein Assay, page 216).

2.8.7.2 Separation of proteins

50μg of each sample was boiled for 5 minutes in SDS-PAGE loading buffer (see page 214).

Electrophoresis of proteins was carried out on vertical SDS-polyacrylamide gels (for details see 8.6.2 Polyacrylamide gel compositions, page 217). The accelerator used was TEMED, and the catalyst was APS (Johnstone and Thorpe, 1987; Hames, 1988).

A 4% to 10% gradient gel was formed using an LKB Bromma 2120 Varioperpex II pump. The top of the resolving gel, which interfaced with the stacking gel, was made even by layering isopropanol over the top of the resolving gel before it polymerised. This was discarded before layering the 2.5% bis-acrylamide stacking gel over the resolving gel. Due to its large pore size the stacking gel had very little sieving effect, ensuring that the proteins all became concentrated into a narrow band at the stacking/resolving gel interface (Johnstone and Thorpe, 1987; Hames, 1988). The gel was left for thirty minutes at room temperature to polymerise.

Samples were loaded and run with Color Markers (Sigma) which were a mixture of proteins of known molecular weight ranging from rabbit muscle myosin at 205 kDa to bovine lung aprotinin at 10.5 kDa. Each marker was conjugated to a different dye such that an estimate could be made regarding the rate of progress of the protein of interest. A constant current of 35mA was applied until the 10.5 kDa protein-dye conjugate band reached the bottom of the gel. To minimise any band distortion, which can occur on passage of an electrical current through the gel, an LKB multicool was used to maintain a constant temperature of 4°C.

The marker used in the western analysis carried out by Dr Bonnie King was a SCA1 cell line from a patient with an expansion in the *SCA1* gene (Coriell Laboratories, Bar Harbor) which was known to consistently produce product bands of suitable size Figure 35, page 191.

2.8.7.3 Transfer

Following electrophoresis, the size-fractionated proteins were transferred to a Hybond membrane (Amersham) using a Bio-Rad Transblot cell®. The filter and gel were equilibrated initially in Western Blot transfer buffer (for details see page 213), then assembled sandwiched in the Transblot cell, and transferred overnight at 100mA at 4°C. After transfer, the filter was washed for five minutes in 1xPBS. This was followed by blocking with Tris buffered saline-Tween (TBS-T) buffer, which is made up of; 0.2% gelatin, 0.1% Tween 20 and 5% dried skimmed milk,

either for one hour at room temperature or overnight at 4°C. The filter was then given a brief wash in 1xTBS-T before applying the primary antibody, 1C2 (non-purified ascites) (Dr Jean-Louis Mandel, INSERM), at 1/5000 dilution in TBS-T and agitating on an orbital shaker for one hour at room temperature. The antibody was removed (and stored at 4°C for re-use). The membrane had three 10 minute washes in 0.5% milk in TBS-T, before incubation with the secondary antibody (goat-anti-mouse conjugated to peroxidase, 1/10000 dilution in blocking buffer) (Jackson Laboratories) for one hour at room temperature with agitation. Three additional, 10 minute washes with 0.5% milk in TBS-T were completed before applying the ECL detection technique (Pierce).

2.8.7.4 Detection system and development

The two ECL detection solutions (exact recipes not given) were mixed in equal quantities such that there was sufficient solution to completely cover the membranes. Any excess wash buffer was drained from the washed blots, which were placed protein-side uppermost on a sheet of SaranWrap™. The prepared ECL solution was poured onto the membrane. This was incubated on the bench for five minutes (making sure that the blots did not dry out). Excess detection reagent was drained off before placing the blot, protein-side down, onto a fresh piece of SaranWrap™. The SaranWrap™ was folded back over the blot to form an envelope. The blots were immediately placed protein side uppermost in the film cassette. The membrane was exposed to a sheet of Kodak X-Omat Imaging film for 30 minutes to allow the signal to develop. The film was developed in a Cronex CX-130 automatic processor (DuPont).

2.8.8 Microbiological manipulations

2.8.8.1 Bacterial media

2.8.8.1.1 Luria-Bertani medium (LB medium) (page 218).

2.8.8.1.2 Luria-Bertani (LB) agar plates

LB agarose plates were prepared at a concentration of 1.2% by the addition of 12g agar per litre of LB, before autoclaving. The mix was allowed to cool to 50°C before pouring approximately 30ml per 90mm plate. Ampicillin (NBL) was added to media and plates at a concentration of 100μg/ml. Both medium and plates were stored at 4°C. Plates were removed from storage two hours before use and placed at 37°C to dry.

2.8.8.2 Competent cell preparation

Competent cells were prepared from JM101 Escherichia coli bacteria (supE, thi, Δ(lac-proAB), [F`, traD36, proAB, lacI9ZΔM15]) (Pharmacia) maintained on M9 thiamine agarose plates as single colony cultures. Cultures were re-plated every six to eight weeks in order to maintain the population as a single clone.

A single colony was inoculated into a 10ml LB broth and cultured overnight at 37°C at 200rpm in an orbital incubator (Gallenkamp). One ml of the overnight culture was then inoculated into 100ml LB broth and incubated under the same conditions for 1.5 to 2 hours, until the optical density at 600nm was between 0.2 and 0.3, thus ensuring that the cell density was not greater than 10^8 cells/ml. At this concentration the bacterial population will be in the exponential part of its growth phase. For operational reasons the bacteria was pelleted by centrifuging the broth as three 40ml aliquots into sterile, ice cold polypropylene tubes (Becton Dickinson) followed by incubation on ice for 20 minutes. Bacteria were then recovered by centrifugation at 5000rpm (JA-20 rotor) for 10 minutes at 4°C. These were gently reconstituted in 50ml of chilled sterile 100mM magnesium chloride and recovered as in the previous step. Following reconstitution, as before, the pelleted cells were incubated on ice with occasional agitation for one hour. The cells were recovered as above and reconstituted in 10ml of ice cold sterile 100mM calcium chloride 15% glycerol. Aliquots were stored at -70°C for a minimum of 24 hours prior to use.

2.8.8.3 Transformations

Transformations were carried out in sterile 10ml Falcon tubes, pre-chilled on ice. To allow for variations in preparations acquired from other laboratories three transformations were performed with nominal amounts equivalent to 1ng, 10ng and 100ng DNA, all made up to 100µl with TE buffer (page 211). An aliquot of competent cells was thawed in the hand. A 100µl volume of competent cells was then added to each DNA sample, swirled gently to mix, and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 45 seconds then rapidly transferred to ice for 2 minutes. 800µl of SOC medium (page 218) was added before incubation in an orbital incubator at 100rpm, 37°C for an hour. 200µl aliquots were plated out on LB ampicillin agar plates and incubated overnight at 37°C.

2.8.8.4 Plasmid preparations

The Wizard™ minipreps DNA purification system (Promega) was used to isolate plasmid DNA from transformed bacterial cells. This is based on the alkaline lysis procedure (Birnboim and Doly, 1979) with lysis of bacteria using sodium dodecyl sulphate (SDS) and NaOH (SDS denatures bacterial proteins and NaOH denatures

chromosomal and plasmid DNA). Neutralisation with potassium acetate causes the covalently closed plasmid DNA to reanneal rapidly whilst most chromosomal DNA and bacterial proteins precipitate with the SDS and form a complex with the potassium which is then removed by centrifugation. Plasmid DNA is further purified by passage through a proprietary chromatography column before elution with TE buffer. Yields of up to 10μg were obtained from three ml overnight cultures. Plasmids were stored as purified DNA at -20°C.

2.8.9 Image recording

2.8.9.1 Photomicrographs

Photographs of histological sections were taken on an Olympus Vanox-S.

2.8.9.2 Immunofluorescent photography

Photographs were taken using a Reichert-Jung Diastar photomicroscope with epifluorescence (Model 2090).

2.8.9.3 Confocal microscopy

The confocal system was an MRC 600 (Biorad).

2.8.9.4 Electron microscopy

The electron microscope was an AEI EM6B.

3. A clinical appraisal of CDRM

3.1 Introduction

The clinical signs of CDRM have been recognised for many years and described by a number of authors (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978; Waxman *et al.*, 1980b; Clemmons, 1989; Clemmons, 1992). Although it was not anticipated that we would find any novel clinical signs it was necessary to perform detailed clinical examinations for selection of dogs for further studies. In addition, it was anticipated that as the present study was dedicated solely to the investigation of CDRM in GSDs it would provide the largest database so far collected on this disorder.

3.2 Aims

The underlying aim of this study was to improve the clinical knowledge of CDRM by regularly examining a large group of affected dogs. The most common presenting sign(s), the age at onset, and whether there was any previously unreported sex predisposition were of particular interest. Additional aims were to tabulate rates of degeneration and note the most commonly seen clinical "syndrome".

3.3 Materials and Methods

3.3.1.1 Selection of cases

Cases were sourced from all over the United Kingdom (northern Scotland to the south of England) but the majority that were examined repeatedly were from the central belt of Scotland (Strathclyde, Ayrshire, Lanarkshire, West Lothian, Perthshire). All dogs with clinical signs suggestive of CDRM were seen *i.e.* pelvic limb ataxia and paresis with a loss of proprioception. In order to increase the number of cases, a request for extra material was made to all veterinary practices in Scotland and the north of England. Cases were also obtained from other specialities, such as the orthopaedic clinic within the veterinary school. The animals in the study were therefore a highly selected group within the general population.

A complete physical and neurological examination was performed on referral to the CDRM clinic at which point they were separated into one of three groups. Cases with non-neurological problems were discarded from the study while those with neurological signs not typical of CDRM were evaluated by the department of neurology, then excluded or included in the study as appropriate. Cases for

inclusion were chosen on the basis of a history and clinical examination which suggested a chronic progressive neurodegenerative disease which affected primarily the thoracolumbar spinal cord. An outline of the procedure, including the further investigations which were carried out as appropriate, is illustrated in Figure 3, page 74.

3.3.1.2 Investigation of cases

The signalment and full history were obtained for all dogs, this included details of other diseases or injuries as well as medication. Family histories and pedigrees were also recorded.

A full physical assessment and neurological examination were carried out as detailed by Oliver and Mayhew (1987) (for details see 2.2 Case Selection, page 32). The following functions were found to be the most useful in the assessment of CDRM.

Gait was assessed initially with the animal moving in a straight line on a flat surface. This was observed from the side, in front and behind the animal. This was followed by assessment of gait during circling or when going up and down stairs.

The wheelbarrow and hopping tests were used primarily to detect weakness of limbs and to highlight any asymmetry between limbs.

Proprioception is the ability of the animal to recognise the location of its limbs without visual input. Neurological tests used to evaluate the sensory modality of proprioception also required adequate motor function by the animal to perform properly. These tests included paw position, reflex step and hip sway tests.

Muscle tone, muscle bulk and the local spinal reflexes (patellar reflex and pedal reflex) were assessed in the pelvic limbs. In the thoracic limbs a similar examination was made, omitting any myotactic reflexes (local reflex arc related to muscle stretch receptors). Such reflexes were omitted on examination of the thoracic limbs as they were known to be inconsistent, even in the normal animal. The perineal reflex and panniculus reflex were also evaluated.

Further investigations were carried out in cases where there was some doubt, following the clinical and neurological examinations, that the dog had CDRM. Nine cases underwent further investigations. These involved spinal radiographs, myelography, CSF analysis and EMG as appropriate (see 2.2.1 Ancillary investigations, page 45).

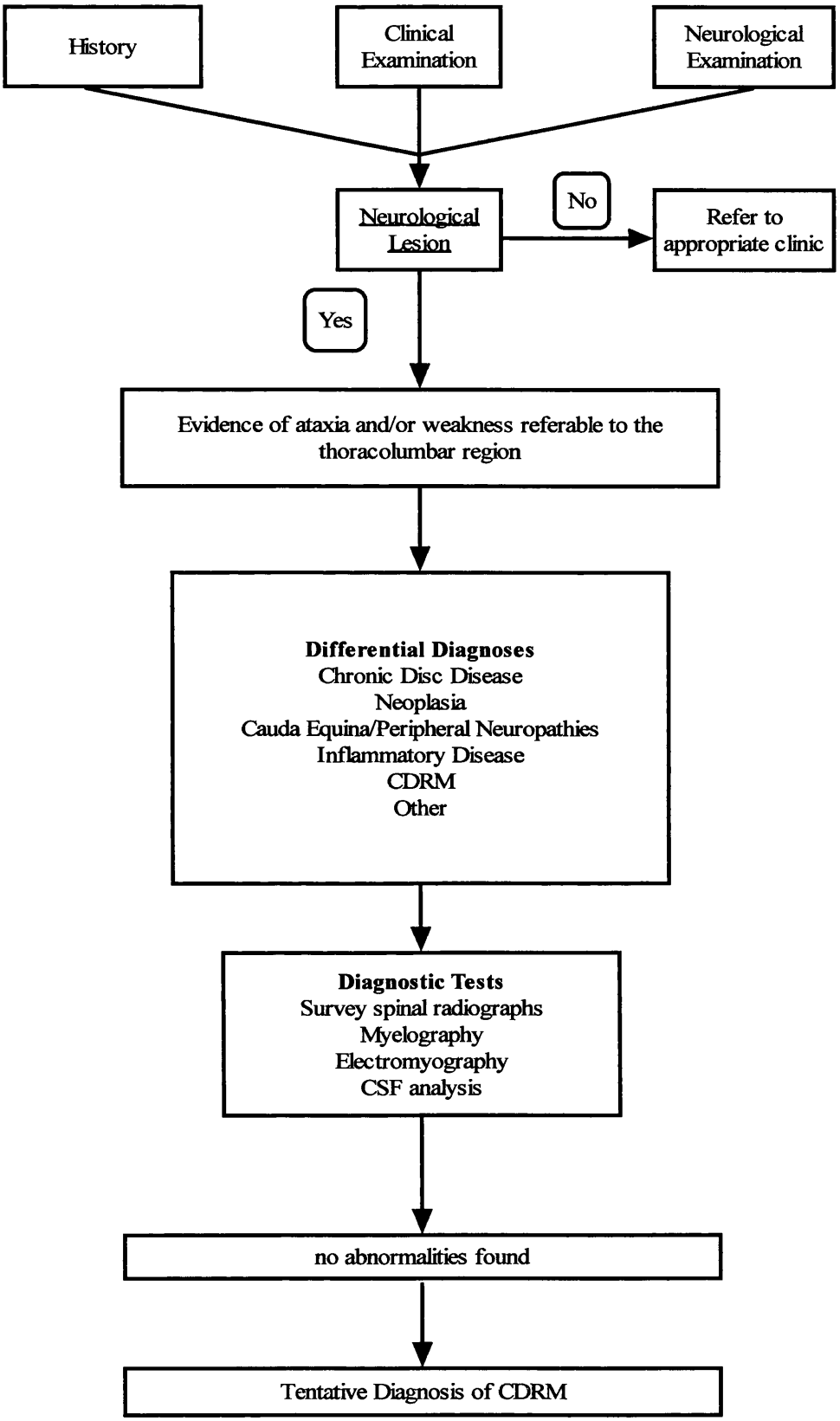


Figure 3. Diagrammatic representation of the complete work up for dogs, with atypical history or clinical signs, before their inclusion in the clinical study.

Based on the results of the neurological examination the lesion was localised within the nervous system. If the localisation was consistent with the picture of CDRM then the dog was included in the study. Inclusion in the study was reassessed with every subsequent re-examination.

Blood samples were obtained at each examination. Routine haematology and biochemistry were checked for 30 of the dogs on first consultation, and a list of parameters measured (for details 2.2.3 Collection of samples, page 47).

3.3.1.3 Therapies

Clemmons (1989; 1992) suggested that a combination of vitamins and essential fatty acids may slow the rate of neurodegeneration in cases of CDRM. These claims remained to be substantiated, however a number of owners decided to give their dogs the therapies listed in Table 8, page 75. Dogs were given either all three therapies simultaneously or none at all.

Therapy	Dose Rate
Evening primrose oil (EPO)	500IU/day
Vitamin B complex	200IU/day
Vitamin E	500IU/day

Table 8. Therapies suggested to owners of dogs with suspected CDRM.

3.3.2 Statistical methods

The difference in distribution of the two genders between the affected and unaffected groups compared to the general referral population was assessed by chi-squared analysis. Significance was set at the 5% level.

3.4 Results

3.4.1.1 Number of cases in the study

The total number of GSDs examined in the study and their subsequent fate are shown in Table 9, page 78. Of these there were two littermate pairs (Dogs 6 and 15; 41 and 47). In addition, the sire of the latter pair (Dog 14) was also included in the study.

Three dogs of other breeds were seen, two Boxers and one Rough collie. These were not included in the clinical study as we were selecting for GSDs.

Breed

The number of GSDs and GSD crosses included in the study are given in Table 10, page 78.

Age at first presentation

Owners were vague about the exact age of onset of clinical signs, so recording of accurate information regarding age of onset was not feasible. Figure 4 (page 79) illustrates age at first presentation for all dogs included in the study.

3.4.1.2 History

Cases were most commonly presented to the referring veterinary surgeon because owners heard scuffing of the nails of the pelvic limbs when exercising the dogs on hard surfaces. Owners reported progression of signs over a six week to six month period before initial presentation. Typically, the signs which developed as the disease progressed were as follows: wearing of the nails became increasingly noticeable and more persistent; all dogs started to have problems turning, developing a tendency for the hindquarters to fall to the outside on a turn. These difficulties were more obvious on slippery surfaces and on stairs. Most cases were still keen to exercise at this stage. Crossing of the pelvic limbs then developed, where one leg would get caught behind the other which often resulted in the dog falling over. Collapsing to one side when walking in a straight line followed. Eventually, after a period of six to 18 months, the dogs were unable to rise to a standing position so pulled themselves along with their thoracic limbs. Only two cases developed signs of thoracic limb involvement, these had both been in a "K-9 Kart®" or "Dogmobile®" for more than 12 months. Of these two cases only one (Dog 160) was subsequently post mortemed.

3.4.1.3 Physical examination

Twenty nine cases had concurrent degenerative joint disease which was being treated with anti-inflammatory drugs. Of these, 11 owners reported some degree of improvement in gait while on anti-inflammatory treatment. One case had evidence of heart disease and was referred to the cardiology clinic for further investigation, the dog was also affected by CDRM. The rest of the cases included in this study were otherwise normal.

3.4.1.4 Neurological examination

The results of the neurological examination performed at first presentation for all dogs examined and last presentation for all dogs seen at the time of euthanasia or within the last six weeks of life are shown in Table 12, page 80. Variations in the quality of the patellar reflex concurrent with the presence of CDRM are presented in Table 13 and Table 14 (page 81).

3.4.1.5 Rates of deterioration

The rate of deterioration was plotted for all dogs seen three times or more in the CDRM clinic. Gait score as detailed in 2.2.2 Gait scoring system, page 46 was used as a measure of deterioration for each case. These results are illustrated in Figure 5 (pages 82-83).

3.4.1.6 Further investigations

Further investigations were carried out on nine cases. Results of these investigations are detailed in Table 15, page 84.

3.4.1.7 Sample treatments

Owners either gave all three suggested therapies or gave none. Numbers of dogs in each group are given in Table 16, page 84. The rate of deterioration was not obviously altered in the cases given the vitamins and evening primrose oil therapies. The rate of deterioration for those dogs on the therapies which were seen three times or more are illustrated in Figure 5, pages 82-83.

3.4.1.8 Blood results

No significant abnormalities were found on routine haematology and biochemistry for any of the dogs tested.

Total number of dogs seen	93	
Proportion of dogs seen then excluded from the study	27	
Proportion of dogs seen which were included in the study	66	
Proportion of included dogs seen once	32	48%
Proportion of included dogs seen twice	19	29%
Proportion of included dogs seen three times	5	8%
Proportion of dogs seen four times	5	8%
Proportion of dogs seen five times	2	3%
Proportion of dogs seen six times	2	3%
Proportion of dogs seen seven times	1	2%

Table 9. Numbers of GSDs and GSD crosses examined in the study with number of examinations.

Number of GSDs	60	(91%)
Number of GSD crosses	6	(9%)

Table 10 Number of GSD and GSD crosses included in the study.

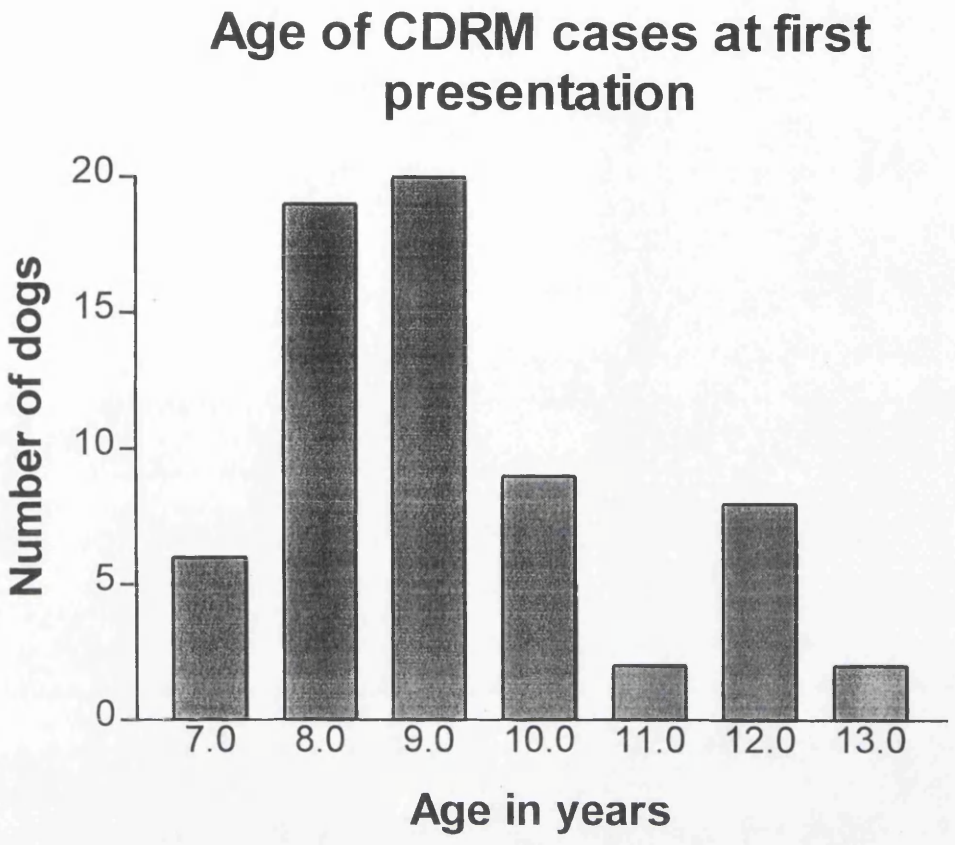


Figure 4. Age of CDRM cases at first presentation (a particular age represents a range of ± 6 months).

Sex	Number of dogs	
Male (intact)	38	(58%)
Male (neuter)	2	(3%)
Female (intact)	15	(23%)
Female (neuter)	11	(16%)
Total	66	

Table 11. Sex of GSDs and GSD crosses included in the study.

Clinical Sign	Number of dogs at first presentation		Number of dogs late in disease*	
Ataxia	66/66	(100%)	38/38	100%
Paresis	64/66	(97%)	38/38	100%
Paw position reflex slow/absent	62/66	(94%)	38/38	100%
Reflex step slow/absent	65/66	(98%)	38/38	(100%)
Sway reflex slow/absent	61/66	(92%)	37/38	(97%)
Worn nails	64/66	(97%)	36/38	(95%)
Scuffed paws	27/66	(41%)	20/38	(53%)
Ulceration	17/66	(26%)	17/38	(45%)
Muscle atrophy	9/66	(14%)	11/33	(33%)
Muscle tone decreased	3/66	(5%)	8/32	(25%)
Panniculus intact	64/66	(97%)	38/38	(100%)
Perineal reflex intact	66/66	(100%)	38/38	(100%)

Table 12. Results of neurological examination of pelvic limbs at first presentation and last presentation before euthanasia.

* 28 of the dogs included in the study were not examined within the last six weeks of life so were excluded from the "late in the disease" column.
The denominator denotes the number of dogs examined for each sign.
The numerator denotes the number of dogs with the mentioned sign.

Response to test	Patellar reflex	
Hypertonic with clonus	1/66	1%
Hypertonic	8/66	12%
Normal	47/66	71%
Hypotonic	8/66	12%
Absent	2/66	3%

Table 13. Results of patellar reflex testing at first presentation

Response to test	Patellar reflex	
Hyperreflexia with clonus	2/37	(5%)
Hyperreflexia	3/37	(8%)
Normal	20/37	(54%)
Hyporeflexia	10/37	(27%)
Absent	2/37	(5%)

Table 14. Results of patellar reflex testing at last presentation for those dogs examined at a late stage in the disease.

The denominator represents the number of dogs tested.
The numerator represents the number with the mentioned response.

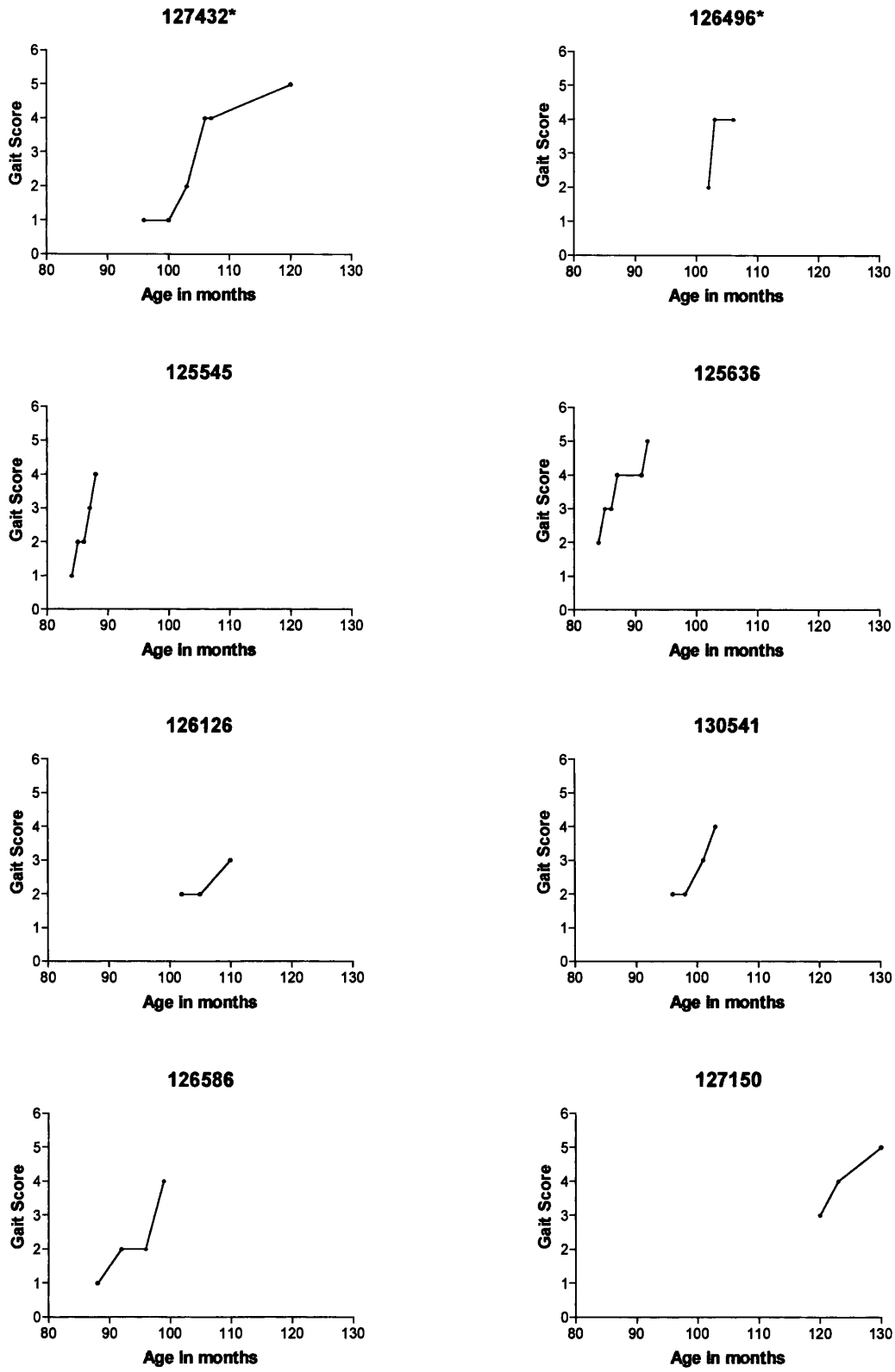


Figure 5a. Rates of deterioration for dogs seen three times or more. All dogs on vitamin therapies except those marked with an asterisk.

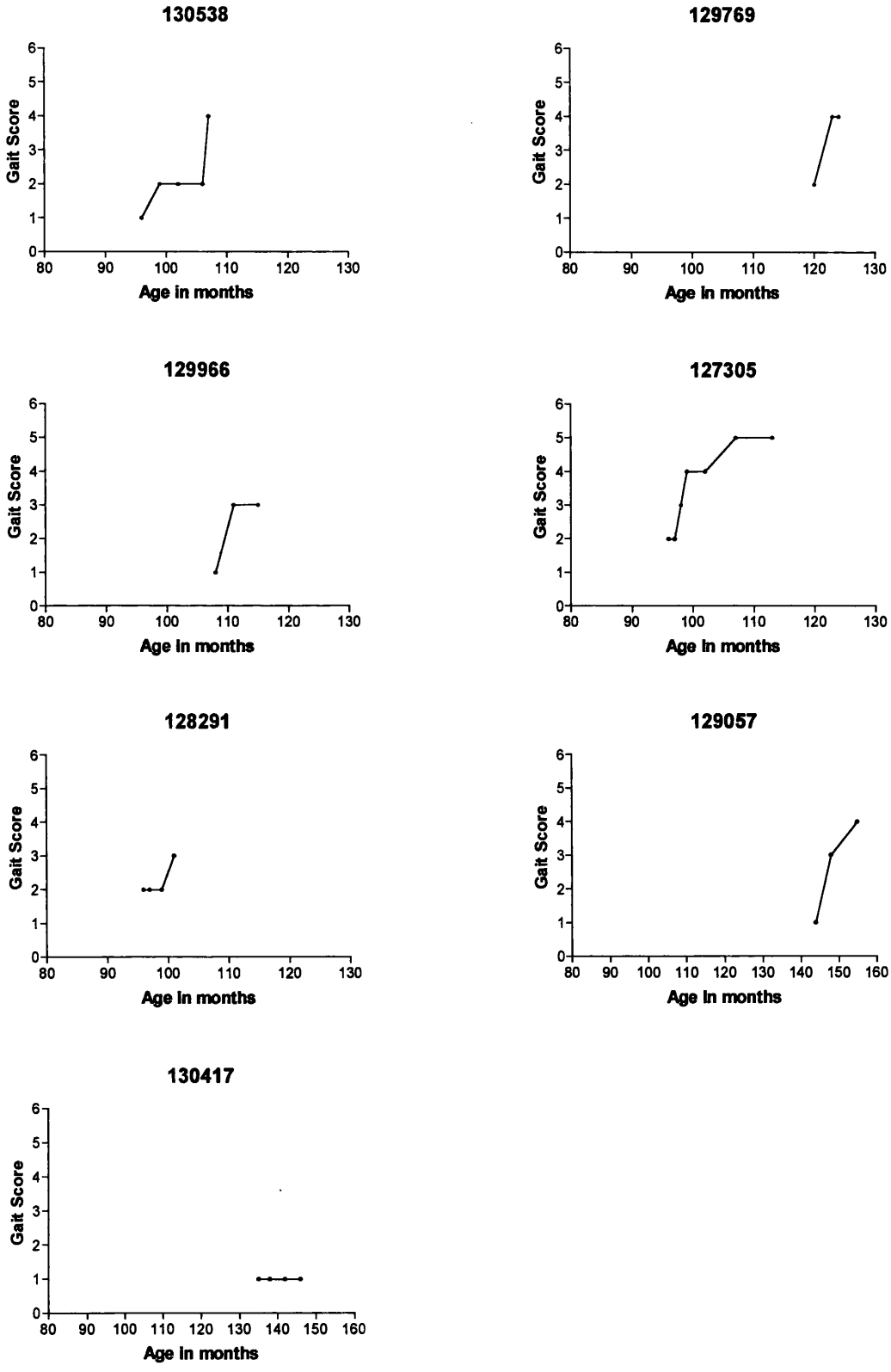


Figure 5b. Rates of deterioration for dogs seen three times or more. All dogs were on vitamin therapies.

Dog ID Number	Survey radiography	Myelography	CSF	EMG
13	HD*/OA**	NAD	NAD	NAD
14	NAD	NAD	NAD	N/A
16	NAD	NAD	NAD	NAD
21	Lumbosacral disease	Lumbosacral disease	NAD	NAD
29	NAD	NAD	NAD	NAD
128	Spondylosis	NAD	NAD	NAD
129	NAD	NAD	NAD	N/A
154	Spondylosis	NAD	NAD	NAD
168	NAD	NAD	NAD	NAD

Table 15. Results for dogs undergoing further investigations.

*Hip dysplasia
**Osteoarthritis of hips
NAD: no abnormalities found N/A: not applicable

Number of dogs on no therapies	6	18%
Number of dogs on vitamin B, vitamin E and evening primrose oil	28	82%

Table 16. Number of dogs on therapies and those on no therapy, as a proportion of the total number of dogs seen more than once.

3.5 Discussion

Although dogs of all breeds which had clinical signs of CDRM were potential candidates for inclusion in the study the nature of the funding meant that we were selecting for GSDs. All dogs included in this study were GSDs or GSD crosses. Previous studies using an unselected population of dogs had 95.5% GSD and GSD crosses (Averill, 1973) and 56% GSDs (Griffiths and Duncan, 1975a). The finding of CDRM in several littermate pairs combined with the acknowledged high incidence of the disease in the German shepherd breed in general suggested that a genetic factor may well be involved in the aetiology of the disease, as previously suggested (Clemmons, 1989).

Age of onset was difficult to calculate accurately as owners were often vague as to the length of time the dog had been demonstrating clinical signs, however most cases were presented at the CDRM clinics between the ages of eight and ten years. Mean age at first presentation was 9.2 years which correlated with previous reports (Averill, 1973; Griffiths and Duncan, 1975a) which had suggested a mean age of onset of 9 years.

Three previous studies (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978) stated that there was no sex predisposition in this disease, although two of them had reported more males affected than females. In the present study a ratio of 1.6 males to females was found, which was not statistically significant by the chi-squared test.

The deterioration in clinical signs seen in the present study followed a similar pattern in all cases and agreed with previous reports (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978). Most cases were presented initially as having a unilateral problem as reported by Griffiths (1975a) and Averill (1973). The clinical signs often continued to be asymmetrical although both pelvic limbs became involved to some extent as the disease progressed. Cases maintained for two years after first developing clinical signs did begin to show thoracic limb signs, usually manifested as stumbling.

Clinical signs were present for six weeks to six months before presentation at the CDRM clinic. The rate of deterioration varied markedly between cases, the most rapidly deteriorating case was euthanased within four months of first examination at the clinic while more slowly progressive cases were followed for up to 12 months before euthanasia at the owners request. This suggested a clinical course of six to 18 months compared to six to 12 months which had been previously suggested

(Clemmons, 1992). A number of cases were euthanased at the owners request while the dogs were still able to walk, however all cases maintained for longer did lose the ability to ambulate due primarily to the severe involvement of the pelvic limbs. The two cases in this study maintained for more than a year were in a "K-9 Kart®" or "Dogmobile®" for the last 12 to 14 months of life.

The data were assessed visually for evidence that the rate of deterioration might be linked to age of onset, however, there did not appear to be any obvious relationship.

The clinical "syndrome" of CDRM was represented by a dog which must have had a history of a gradually progressive pelvic limb gait problem and worn nails but no evidence of spinal pain. On physical examination the dog was likely to be otherwise normal (Averill, 1973; Griffiths and Duncan, 1975a). The gait abnormality was due to both ataxia and paresis, the ataxia being more prominent than the paresis, as reported by Griffiths (1975a), Averill (1973) considered the distinction to be unclear. On neurological examination all cases had altered conscious proprioception as evidenced by the paw position, reflex step and hip sway reflexes initially becoming sluggish then, as the disease progressed, disappearing completely. Panniculus and perineal reflexes were almost always intact. Any cases found to have restricted panniculus reflex during the course of the study underwent radiography and myelography (two cases). In this instance neither had any evidence of cord compression, so were included in the study. Slight decreases in muscle tone were found, usually unilaterally, in a small number of dogs. The proportion of animals with decreased muscle tone increased as the disease progressed. This was in contrast to a previous report which had suggested that muscle tone remained normal (Griffiths and Duncan, 1975a).

Additional signs of importance were a lack of any spinal pain and no evidence of faecal or urinary incontinence (except very late in the course of the disease). The clinical signs had been well documented in previous studies (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978; Waxman *et al.*, 1980b; Clemmons, 1989; Clemmons, 1992).

The clinical signs indicated both motor (paresis) and sensory deficits (abnormal proprioception) and suggested that the major abnormalities occurred within the T3 to L3 region of the spinal cord. None of the cases in the present study showed any evidence of focal localisation of signs within this region of the spinal cord. Even the two cases which had a restricted panniculus reflex had no evidence of a focal lesion on further investigation.

Ataxia indicated the failure of muscle coordination which resulted in staggering and irregular muscular movements and resulted from disruption to the sensory pathways responsible for proprioception.

Paresis indicated a deficit of voluntary movements and resulted from disruption of the voluntary motor pathways. The clinical deficits suggested that the neuropathology should involve both the motor pathways and the sensory tracts which dealt with proprioception. These are discussed in 5 An investigation into the pathology associated with CDRM, page 112.

Asymmetry of signs had been reported by Griffiths and Duncan (1975a) and this finding was supported by this study. Many dogs had one pelvic limb more severely affected than the other, although as the disease progressed both pelvic limbs did invariably become involved.

The vast majority of cases in this study had an intact panniculus reflex despite the evidence that the lesion(s) affected cord segments which mediated the reflex (C8-L1). The probable explanation is that the specific fibres or interneurones concerned with the panniculus reflex were not involved in the pathology of CDRM.

Perineal reflex was normal which indicated that there was functional integrity of the sacral spinal cord segments and sacral nerve roots.

Some variation was found in the patellar reflex both between individuals and in the same animal at different stages in the disease. In the majority of instances the reflexes were present, whether normal or exaggerated. This would be consistent with a major pathology within the regions of cord (T3-L3), as indicated above. A small proportion of affected dogs (32%) showed decreased or absent patellar reflex, usually unilaterally. There were a number of possible explanations for this finding. In the cases where there was no other evidence of a LMN lesion, such as atrophy, it was probable that the defect which resulted in the hyporeflexia occurred within the afferent side of the reflex. This had been suggested previously (Griffiths and Duncan, 1975a). Three of the dogs (Dogs 1, 2, 16) with decreased or absent patellar reflexes also had some muscle atrophy late in the disease. The most likely explanation for this was a secondary atrophy due to decreased levels of physical activity as a result of the gradually progressive ataxia and paresis. Another possible explanation was that the spinal cord changes had extended caudal to L4 leading to atrophy due to LMN involvement, this was not borne out by the pathology found in these cases (5 An investigation into the pathology associated with CDRM, page 112) as there was no significant loss of ventral grey matter neurones in the caudal lumbar and sacral segments.

Four of the cases radiographed during this project showed abnormalities, two had spondylosis, one lumbosacral disease and the fourth hip dysplasia with secondary osteoarthritis. These findings would be expected in a population of middle-aged and old GSDs. Spondylosis was a common finding during radiographic or post mortem examination of the vertebral column. It was characterised by the formation of bony spurs and bridges at the intervertebral spaces and occurred most commonly in the caudal thoracic and caudal lumbar vertebrae (Oliver and Lorenz, 1983). This condition is rarely the cause of neurologic signs although it may occasionally result in spinal pain. Neither of the dogs with spondylosis had any evidence of compression of the spinal cord. Disease in the lumbosacral region may be due to a number of causes such as intervertebral disc degeneration, discospondylitis or neoplasia. Signs resulting from disease in this area may include pelvic limb gait abnormalities, lameness or LMN neurological deficits (Palmer and Chambers, 1991; Wheeler, 1992; Ness, 1994). The lower urinary tract, tail and anus may all be affected and pain is a relatively common sign. A likely diagnosis of these conditions may be made on recognition of the clinical signs described here and on a careful clinical examination which may pinpoint the focus of pain, followed by survey radiography and myelography. The dog in this study which had signs of disease in the lumbosacral region had no evidence of associated pain. It was concluded that the changes seen on radiography and myelography were incidental and the dog was included in the CDRM study.

In the case with hip dysplasia and secondary osteoarthritis these conditions were contributing to, and complicating the assessment of, the pelvic limb gait. However, there were neurological deficits not attributable to either of these conditions which suggested concurrent CDRM.

Routine haematology and biochemistry results were normal in all cases, which suggested the lack of any detectable systemic disease which could have caused the neurological signs in CDRM. In addition, CSF samples from eight of the dogs were analysed and no abnormalities were found, in agreement with a previous report (Griffiths and Duncan, 1975a).

Visual analysis of the rates of deterioration of dogs given vitamin E, vitamin B and evening primrose oil, showed no obvious difference to the rates of deterioration of those in the "untreated" group. Ideally the numbers of dogs in these two groups would have been more balanced. As this was not a clinical trial of any kind, and as there was no known effective treatment for this condition, the decision of whether or not the dogs were given the supplements was left to the owner. As the owners represented a highly motivated population the inevitable result was that the vast

majority of cases seen were on the dietary supplements (26/34). A further problem with analysing this information was the fact that dogs with CDRM do deteriorate such that they have periods of deterioration interspersed with periods of no apparent deterioration. Thus in the short term, the supplements could have appeared to slow down the degeneration depending on the precise timing of the initiation of therapy. However, as the dogs were being objectively reassessed at GUVS on a regular basis throughout the clinical course of disease any such discrepancies should have been minimised.

As a result of the information collected in this clinical study, the earliest and most useful clinical signs seen in CDRM were scuffed nails and deterioration in gait on circling and going down stairs. Very early cases were seen which had the gait abnormalities described above but only went on to develop evidence of conscious proprioceptive deficits at subsequent examinations. Repeated clinical examinations, in the absence of further investigations, were useful in increasing the index of suspicion that a dog with pelvic limb ataxia and paresis had CDRM. The rate of deterioration varied between individual animals. It was not possible to accurately predict the rate of deterioration for any one animal. Individual dogs tended to either deteriorate rapidly throughout the course of disease or deteriorate more slowly throughout. However, all affected dogs had periods of deterioration in clinical signs interspersed with periods when there was no apparent deterioration.

4. An investigation into the association of serum vitamin E concentrations with CDRM

4.1 Background information

Vitamin E was first identified seventy years ago although its function had only recently been established.

4.1.1 Forms and structure

Vitamin E was the generic term given to a group of lipid soluble compounds which were derivatives of tocopherol which had a saturated side chain and tocotrienol which had an unsaturated side chain (Southam *et al.*, 1991). There were eight naturally occurring forms of vitamin E; α,β,γ and δ-tocopherol and α,β,γ and δ-tocotrienol (Kayden and Traber, 1993b). It was known that α-tocopherol was in higher concentrations in the plasma and tissues than γ-tocopherol despite dietary γ-tocopherol concentrations being much higher than α-tocopherol. This suggested that a mechanism existed which discriminated between the various forms of vitamin E. Experiments had shown that α and γ tocopherols are absorbed from the intestine equally and transported by chylomicrons in the plasma. The discrimination arose in the liver, where α-tocopherol was preferentially secreted in association with nascent very low density lipoproteins (VLDL), while γ-tocopherol taken up by the liver was eventually excreted in bile (Arita *et al.*, 1995). Of the eight naturally occurring forms, α-tocopherol had the greatest biological activity due to its selective concentration in the liver and subsequent secretion in blood relative to the other compounds and accounted for over 90% of the vitamin E in tissues (Arita *et al.*, 1995).

4.1.2 Regulation of plasma α-tocopherol

Plasma α-tocopherol concentrations were maintained within narrow limits due to the activities of α-tocopherol transfer protein (α-TTP), also called tocopherol-binding protein. It had been demonstrated that in human patients who demonstrate a lack of transfer protein, plasma α-tocopherol concentrations fell rapidly, which suggested that the protein was necessary to maintain minimal levels (Ouahchi *et al.*, 1995). In addition, in normal patients supplementation with up to 100 times the normal daily intake (15mg) of α-tocopherol did not result in a proportional increase in plasma α-tocopherol concentrations (Kayden and Traber, 1993b). Vitamin E, unlike the other fat soluble vitamins, was not toxic if given in high doses, possibly because it was not stored in the liver. Excess absorbed tocopherols were readily excreted in bile; α-TTP was necessary both for salvage of α-tocopherol to prevent excretion, and to facilitate incorporation into VLDL for secretion into plasma.

4.1.3 Mechanism of action

In vitro, and probably *in vivo*, α-tocopherol acted as an antioxidant. Antioxidants were defined as substances which slow down or prevent oxidation of vulnerable substrate. α-tocopherol was a lipid-soluble, chain-breaking, antioxidant or free radical scavenger which reacted with lipid peroxyl radicals to yield a relatively stable lipid hydroperoxide, thus it protected against membrane lipid peroxidation (Cummings *et al.*, 1997) and appeared to be the only significant lipid-soluble, chain-breaking, antioxidant *in vivo*. Peroxidation of membrane lipids had several consequences which included inactivation of membrane enzymes, decreased membrane fluidity and formation of lipoperoxide clusters which eventuated in membrane pores for Ca^{2+} influx. Vitamin E could terminate free-radical generated chain reactions by scavenging peroxyl radicals formed by the action of oxygen-derived free radicals on unsaturated fatty acids (Mascio *et al.*, 1991). In addition it might also protect other molecules, such as membrane proteins, from oxidation. It had also been suggested that the phytyl side chain of the tocopherol molecule could interact with the arachidonyl fatty acid chains of membrane phospholipids and so facilitated molecular packing and the maintenance of membrane stability (Southam *et al.*, 1991). The CNS was reported to be particularly susceptible to oxidative injury. This was due to the high level of neuronal oxidative metabolism and the associated generation of reactive oxygen species combined with the abundance of membrane vulnerable to peroxidative damage and the lack of regenerative capability in neurones (Cummings *et al.*, 1997).

4.1.4 Neurodegenerative disorders associated with experimentally induced vitamin E deficiency

Experimental animals which included monkeys (Nelson *et al.*, 1981), rats (Pentschew and Schwarz, 1962; Southam *et al.*, 1991) and dogs (Hayes *et al.*, 1970; van Vleet, 1975) fed a vitamin E-deficient diet exhibited a wide range of pathological states involving the haematopoietic, vascular, nervous, musculoskeletal and reproductive systems. A number of specific neurological changes had been found in both monkeys and rats (Pentschew and Schwarz, 1962; Nelson *et al.*, 1981; Southam *et al.*, 1991).

Prolonged and severe experimental vitamin E deficiency in rats resulted in a neurological syndrome which was characterised by muscle weakness, tremor, ataxia and hyperaesthesia. Morphological studies had shown that this was associated with the development of axonal dystrophy in the cranial parts of the dorsal columns and the gracile and cuneate nuclei with secondary loss of myelin and accompanying

Wallerian-like fibre degeneration *i.e.* changes which are typical of a central-peripheral distal axonopathy (Pentschew and Schwarz, 1962; Southam *et al.*, 1991).

Progressive neuropathological lesions had been seen in vitamin E deficient rhesus monkeys (Nelson *et al.*, 1981). In this study a group of monkeys were maintained on a vitamin E deficient diet for over two years. The most dramatic CNS change which resulted from this diet was the concomitant loss of axons and myelin sheaths in the dorsal columns accompanied by mild, fibrillary astrocytosis.

4.1.5 Naturally occurring neurodegenerative disorders associated with vitamin E deficiency

The specific mechanism of the neurological abnormalities seen with vitamin E deficiency had not been absolutely established. It had been suggested that these could be the result of damage to the function of mitochondrial and other intra-axonal membranous structures which would have interfered both with fast anterograde transport and "turnaround" so leading to a distal degeneration of axons (Southam *et al.*, 1991).

4.1.6 Equine neurodegenerative disorders associated with vitamin E deficiency

Equine degenerative myelopathy (Liu *et al.*, 1983; Scarratt *et al.*, 1985), equine degenerative myeloencephalopathy (EDM) (Dill *et al.*, 1990; Blythe and Craig, 1992a; Blythe and Craig, 1992b) and neuroaxonal dystrophy (Baumgartner *et al.*, 1990) were all conditions which had been tentatively linked to vitamin E deficiency, although some workers had suggested that horses with, for example, EDM did not have significantly different serum vitamin E concentrations compared with control animals (Dill *et al.*, 1989). These have been considered in detail on pages 24-26.

Horses with equine motor neurone disease (EMND) had markedly depressed blood levels of vitamin E (Summers *et al.*, 1995). EMND was a neurodegenerative disease of the somatic LMNs which resulted in a syndrome of diffuse neuromuscular disease in the adult horse characterised by weight loss, muscle atrophy, increased periods of recumbency and fasciculations of the proximal limb musculature. The neurodegenerative changes involved the cell bodies of the bulbospinal motor neurones and, to some extent, the primary sensory neurones. A number of aetiological factors had been associated with this condition. Vitamin E deficiency was thought to lead to oxidative injury to motor neurones which caused profound extensor weakness of the major weight-bearing muscle masses especially

the triceps and quadriceps (Mayhew, 1994; De la Rúa-Domènech *et al.*, 1997; Valentine *et al.*, 1997).

4.1.7 Human diseases associated with vitamin E deficiency

Vitamin E deficiency was very uncommon in man but could occur either due to chronic fat malabsorption, as occurred in atresia of the bile ducts and other severe forms of chronic cholestasis in children, or secondary to a number of genetic disorders. Abetalipoproteinaemia produced the most severe vitamin E deficiency. In these conditions the neurological signs attributable to vitamin E deficiency formed part of a complex multisystemic disorder which resulted from multiple defects of other fat-soluble vitamins and essential factors (DiDonato, 1995). The end result in all these conditions was vitamin E deprivation of a number of organs, which included the CNS and PNS. The resulting neurological deficit was a peripheral neuropathy caused by the dying-back of large diameter axons. All patients demonstrated decreased vibration sense. A number also developed areflexia and ataxia.

There was one distinct clinical and genetic entity which had been recognised and resulted in spinocerebellar degeneration. This condition, previously known as familial isolated vitamin E deficiency (FIVED), was first recognised by Burck *et al.*, (1981), and was subsequently called ataxia with vitamin E deficiency (AVED). AVED is an autosomal recessive neurodegenerative disease which mapped to chromosome 8q13. In this condition the intestinal absorption of dietary α-tocopherol was normal, as was incorporation into chylomicrons but hepatic secretion into the blood was impaired, which resulted in plasma vitamin E concentrations which were approximately 10% of normal. This was thought to be due either to the lack of α-tocopherol transfer protein (α-TTP) or the impaired ability of α-TTP to incorporate α-tocopherol into nascent VLDLs which were then secreted by the liver (Arita *et al.*, 1995), the process by which efficient recycling of plasma vitamin E occurred in normal individuals. Mutations in the α-TTP gene in AVED patients demonstrated that vitamin E deficiency was the primary cause of the neurodegenerative process (Ouahchi *et al.*, 1995).

Abetalipoproteinaemia patients had been shown to have a defect in the gene for the microsomal triglyceride transfer protein, an essential protein for the synthesis of VLDL in the liver. Age of onset was generally between four and 18 years, patients showed progressive development of cerebellar ataxia, dysarthria, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss and positive Babinski sign. Patients with AVED had almost undetectable plasma vitamin E concentrations

if they consumed a normal diet. If they were given vitamin E supplements of 400-1200IU/day they were able to maintain normal plasma α-tocopherol concentrations. Effective vitamin E supplementation required multiple dosing throughout the day. Such supplementation of vitamin E did seem to override the rapid elimination of the vitamin and appeared to prevent progression of the disease.

4.2 Justification for the study

A number of neurodegenerative diseases which had been identified in other species had been associated with low circulating vitamin E concentrations, in some conditions treatment with high doses of vitamin E from an early age could reduce the rate of neurodegeneration (Blythe and Craig, 1992b; Ouahchi *et al.*, 1995). Several authors (Williams *et al.*, 1984; Williams *et al.*, 1985) had suggested that the propensity of GSDs for small intestinal malabsorption conditions may have been related to the relatively common occurrence of CDRM in this breed, due to a deficiency in vitamin absorption. In addition Williams *et al.* (1985) conducted a study on a small number of GSDs with CDRM (n=7) and concluded that GSDs had a slightly lower serum vitamin E concentration than other dogs. For these reasons it was appropriate to look at serum vitamin E concentrations in three populations of dogs: (a) GSDs affected by CDRM (b) GSDs unaffected by CDRM and (c) dogs of other breeds unaffected by CDRM. The aims of this study were to answer the following questions (i) do GSDs have significantly lower serum vitamin E concentration than the general canine population (ii) do GSDs with CDRM have a significantly different mean serum vitamin E concentration compared with GSDs unaffected by CDRM and (iii) is there any significant difference in "between dog" variation between GSDs with CDRM and GSDs unaffected by CDRM.

Although the clinical signs of CDRM were restricted to the CNS, it was felt appropriate to measure serum concentrations of the vitamin as this technique had been used successfully in other species. For example, in conditions such as vitamin E related peripheral neuropathy in humans in which vitamin E concentrations from nerve biopsies had not been available serum concentrations were found to be significantly decreased (Kayden, 1993a).

4.3 Materials and Methods

4.3.1 Collection, preparation and analysis of serum vitamin E concentrations

Blood samples were collected and serum prepared as described in 2.2.3 Collection of samples, page 47. Serum samples were stored at -70°C then processed at SAC, Auchincruive as described in 2.2.3.1 Measurement of serum vitamin E, page 47. The preliminary investigation involved six samples from dogs unaffected by CDRM and nine samples from affected dogs. A more detailed study was then instigated. This involved collection of serial samples from a control population of 26 non-GSDs (including two GSDXs) and 20 unaffected GSDs. The age range in the two control populations was two years to 15 years. The CDRM cases were sampled on each visit to the clinic. An attempt was made to sample all dogs at the same time of day following an overnight fast. Details of all dogs used in the study are given in Table 1, pages 33-44. The dogs used in this study were diagnosed on clinical signs and history, many of them were not post mortemed, therefore definitive diagnosis was not always possible. Of the CDRM cases tested more than once, nine were on vitamin E therapy (as well as vitamin B and EPO) following collection of the initial blood sample while three received no therapy.

4.3.2 Statistical methods

The preliminary investigation was analysed using the two-sample t-test. Significance was set at the 5% level.

For the further investigation two methods of statistical analysis were undertaken. The mean concentrations of vitamin E in GSDs with CDRM, GSDs unaffected by CDRM and non-GSDs unaffected by CDRM were compared using one way analysis of variance. Analyses were undertaken on variation in vitamin E concentrations within dogs. This provided a measure of fluctuation in repeat determinations from the same dog in different groups using estimates of components of variance. Similarly, where possible, variation between dogs in different groups was compared. The statistical techniques used are detailed in Clarke and Kempson, 1997.

4.4 Results

4.4.1 Preliminary investigation

Analysis of vitamin E concentrations for dogs in the preliminary investigation using the two-sample t-test, suggested that there was no significant difference between vitamin E concentrations in GSDs with CDRM and non-GSDs unaffected by CDRM, however a much larger study was required to confirm this finding. These data are presented in Table 17, page 102.

4.4.2 Further investigation

4.4.3 Mean vitamin E concentrations in different groups

Table 18 (page 103) shows the vitamin E concentrations for dogs in the three groups *i.e.* GSDs with CDRM, GSDs unaffected by CDRM and non-GSDs unaffected by CDRM. Clearly the means suggested that GSDs with CDRM have higher concentrations of vitamin E than GSDs unaffected by CDRM and also non-GSDs unaffected by CDRM. Statistical analysis for differences amongst means, shown in Table 18b (page 104), indicated a significant difference ($p=0.026$) and the Newman-Keuls multiple range indicated that GSDs with CDRM (mean 46.64μmoll^{-1}) had significantly higher concentrations than non-GSDs unaffected by CDRM (mean 34.15μmoll^{-1}) but not significantly greater than GSDs unaffected by CDRM (mean 37.32μmoll^{-1}). Data were available on a further group of two non-GSDs with CDRM, both GSDXs, (mean 26.30μmoll^{-1}) which was not included in any of the statistical analyses as the sample size was insufficient.

4.4.4 Variation in vitamin E concentrations in different groups

In addition to examining mean concentrations of vitamin E it was instructive to examine variation in vitamin E concentrations both between and within dogs. This was possible using components of variation analyses.

Table 19a (page 105) investigated the variability in vitamin E concentrations in GSDs with CDRM. In view of the repeat determinations in individual dogs, it was possible to estimate two sources of variation. Table 19b (page 106) illustrates the procedure used to estimate variation within dogs and variation between dogs (Clarke and Kempson, 1997). The variation within dogs provided a measure of spread of measurements collected from one dog over a period of time and the standard deviation was found to be 23.7μmoll^{-1}. In contrast the variation between dogs provided an estimate of variation from one dog to another and the standard

deviation was found to be 18.0μmoll^{-1}. These findings suggested that for the group of GSDs with CDRM the variation from one measurement to another on the same dog was likely to be greater than the variation from one dog to another. This could, however, have been an artefact of the presence of disease.

In contrast, Table 20a and 20b (pages 107 and 108) show the results of estimates for within and between dog variation for GSDs unaffected by CDRM. The standard deviation for within dog variation was 7.7μmoll^{-1} and the standard deviation for between dog variation was 10.4μmoll^{-1} suggesting that dog to dog variation in this group was greater than within dog variation.

For dogs in the general canine population, Table 21a and 21b (pages 109 and 110) indicated that the levels of between and within dog variation were small. The standard deviation for within dog variation was 6.9μmoll^{-1} and the standard deviation of between dog variation was 8.5μmoll^{-1} suggesting that dog to dog variation was greater than within dog variation.

4.4.5 Comparison of within and between dog variation between groups

Table 22 (page 111) summarised the estimates of components of variation obtained for each group. The F-test in Table 23 (page 111) clearly indicated that within dog variation concentrations were different for GSDs with CDRM and GSDs unaffected by CDRM. The GSDs with CDRM had a significantly higher ($p=0.003$) within dog variation than those unaffected by CDRM. This suggested that the presence of disease could have been producing large changes in vitamin E levels from one measurement to another.

Similarly, Table 24 (page 111) indicated that the differences in vitamin E concentrations from one dog to another in the group of GSDs with CDRM were significantly greater ($p=0.009$) than from one dog to another in the group of GSDs unaffected by CDRM. Once again this suggested that GSDs with CDRM had more variable concentrations than GSDs unaffected by CDRM.

Although not shown, it was evident from the low estimates of within and between dog variation for non-GSDs unaffected by CDRM (shown in Table 22, page 111) that GSDs with CDRM had significantly higher within and between dog variation in concentrations than the general canine population.

4.5 Discussion

Inspection of mean vitamin E concentrations in the different groups suggested that these were significantly higher in GSDs with CDRM than for the general canine population but not significantly higher than in GSDs unaffected by CDRM. Estimates of components of variance indicated that fluctuations were higher in dogs with CDRM compared to dogs without CDRM, irrespective of breed. Fluctuation from dog to dog was higher in GSDs with CDRM compared to GSDs without CDRM. It was not possible to compare fluctuations from dog to dog for non-GSDs with and without CDRM.

It must be emphasised that the dogs included in the two control populations were age-matched as closely as was possible for a study based on clinical cases, however a number of the controls were under six years of age. Additionally, many of the dogs in the group of other breeds unaffected by CDRM were owned by veterinary surgeons and thus fed on high quality complete diets. In addition, the number of dogs in each group varied. The statistical analyses were undertaken such that the discrepancies in group size were taken into account. These factors did not invalidate the results but had to be considered when conclusions were drawn from the findings.

The results from this investigation suggested that, contrary to previous reports of comparable neurodegenerative disorders in other species (Blythe and Craig, 1992b; Mayhew, 1994; Ouahchi *et al.*, 1995; Arita *et al.*, 1995) where serum vitamin E concentrations were low, and to reports of decreased vitamin E concentrations in GSDs with CDRM compared to unaffected dogs (Williams *et al.*, 1985) (i) GSDs did not have significantly lower serum vitamin E concentrations compared with the general canine population. In contrast, the vitamin E concentrations were found to be significantly higher in GSDs with CDRM than in the general canine population. Furthermore, (ii) GSDs with CDRM did not have significantly different mean serum vitamin E concentrations compared with GSDs unaffected by CDRM and (iii) the GSDs with CDRM had a significantly higher within dog variation than GSDs unaffected by CDRM. Although many of the CDRM cases were given vitamin E therapy this was started after collection of the initial blood sample which was used for calculation of the mean, so the initial values obtained did represent untreated dogs.

Hayes (1970) sampled a wide range of dog breeds and found vitamin E concentrations in the normal GSDs were within the normal range for the general canine population. However, Williams *et al.* (1985) investigated a small number of

GSDs with CDRM (n=7) and found that they had a slightly lower serum vitamin E concentration than control dogs. These latter authors consider that the GSDs with CDRM also had an enteropathy which could cause improper absorption of essential nutrients. Williams (1984) investigated the possible involvement of vitamin B_{12} deficiency but ruled this out as a possible cause of CDRM. Serum vitamin E concentrations, when measured, were lowered in neurological disorders in other species which had been attributed to vitamin E deficiency (Mayhew *et al.*, 1987; Baumgartner *et al.*, 1990; Blythe *et al.*, 1991a; Blythe and Craig, 1992a). Work done in other species had suggested that serum vitamin E concentration was decreased quite considerably before any associated disease occurred. For example, there was at least a three fold drop in serum vitamin E concentration before it was associated with clinical disease in EMND (de la Rua-Domenech *et al.*, 1997) and a ten fold decrease before AVED occurred in humans (Doerflinger *et al.*, 1995).

The higher fluctuation found in GSDs with CDRM compared with GSDs without CDRM may have been due in part to the fact that many of the CDRM cases were on vitamin E therapy. However, of the 12 GSDs with CDRM which were sampled more than once, eight showed an increase in serum vitamin E concentration with time while four showed a decrease. Of the four dogs which showed a decrease in vitamin E concentration, two were on the vitamin therapies and two were not. This may have been a reflection of inconsistent treatment of particular cases or an indication that serum vitamin E levels were not significantly affected by the level of therapy given (500IU/day).

Serum vitamin E concentration was not the most accurate indicator of vitamin E concentration in the body. In man serum vitamin E represented only 1% of the total vitamin E concentration in the body. In this species the vitamin E concentration in adipose tissue was the most closely correlated with that in the nervous system. Also in man, measurement of RBC fragility had been used as a functional test to substantiate vitamin E deficiency (Blythe *et al.*, 1991b). The conclusion from this information had to be that determination of serum vitamin E concentrations was useful as a screening test, but was unlikely to be an accurate indicator of vitamin E concentrations in the CNS of the dog.

Several authors (Beech, 1987; Blythe and Craig, 1992a) had argued that serum vitamin E level could not be used to diagnose EDM, as horses on the same farms as affected animals occasionally had equally low serum vitamin E levels with no associated clinical signs. In addition, a normal level at the time of measurement did not eliminate the possibility of an earlier deficiency during a critical period of development. This was especially relevant to EDM which had an early onset but

was less likely to be of relevance in the case of a late onset disease such as CDRM. It was unlikely that any developmental abnormality caused by deficient nutrition in the first year of life would take six years or more to manifest itself. Further proof of this was provided by dogs which had been fed an experimental vitamin E deficient diet at weaning, these animals developed clinical signs within 40 to 60 days (van Vleet, 1975) which consisted of progressive muscular weakness, subcutaneous oedema, anorexia, depression, dyspnoea and eventual coma. Associated pathological changes were extensive skeletal muscle degeneration, focal subendocardial necrosis in the ventricular myocardium, intestinal lipofuscinosis and renal mineralisation. These findings suggested that dogs rapidly show multisystemic clinical signs when fed a vitamin E deficient diet. None of the dogs used in the present study had evidence of any of these systemic effects.

The data presented in this chapter, in the light of findings associated with vitamin E deficiency and disease in the dog and other species, suggested that deficiency of vitamin E is unlikely to be a primary factor in the aetiology of CDRM. In addition, the results have produced the interesting finding that vitamin E concentrations were higher in the GSDs with CDRM tested than in the unaffected dogs of other breeds tested, which were used to represent the general canine population. Measurement of the concentration of vitamin E in the CNS itself would provide a more accurate indication of whether or not abnormal vitamin E metabolism at the subcellular level was likely to be involved in the aetiology of CDRM.

	Dogs with CDRM	Dogs without CDRM
	53.4	48.5
	33.8	58.2
	18.1	24.8
	52.7	75.7
	28.5	57.7
	39.7	32.7
	27.2	
	76.8	
	42.3	
mean	41.39	49.60
stdev	17.67	18.56
count	9	6

Table 17a. Preliminary serum vitamin E concentrations ($\mu moll^{-1}$) in dogs with CDRM and dogs unaffected by CDRM.

t-statistic	df	p
-0.86	10	0.41

Table 17b. Two-sampled t-tests results comparing serum vitamin E concentrations between dogs with CDRM and dogs unaffected by CDRM.

	GSD with CDRM	Group GSD without CDRM	Non-GSD without CDRM
	51.0	20.4	27.2
	106.0	19.8	32.9
	45.1	29.7	37.1
	23.6	52.6	26.1
	30.3	29.6	14.0
	48.3	60.6	45.0
	88.4	38.1	28.1
	44.4	48.6	29.0
	32.7	47.5	43.2
	36.9	23.2	37.6
	35.1	22.6	33.3
	29.1	26.3	19.7
	38.7	44.4	44.3
	99.8	50.5	39.7
	59.2	32.4	74.2
	46.9	47.5	47.3
	46.3	55.7	26.6
	33.5	18.9	28.7
	45.2	40.9	6.7
	33.5	37.0	38.1
	56.3		49.9
	20.5		30.0
	38.2		34.6
	35.3		33.7
	41.7		33.1
			27.7
mean	46.64	37.32	34.15
stdev	21.62	13.17	12.79
count	25	20	26

Table 18a. Vitamin E concentrations ($\mu moll^{-1}$) in GSDs with CDRM, GSDs without CDRM and non-GSDs without CDRM.

Source	df	ss	ms	F	p
Group	2	2115	1057	3.86	0.026
Error	68	18605	274		
Total	70	20720			

Table 18b. Results of one-way analysis of variance comparing mean Vitamin E concentrations in GSDs with CDRM, GSDs without CDRM, and non-GSDs without CDRM. Follow-up Newman-Keuls multiple range testing (Clarke and Kempson, 1997) indicated that the mean Vitamin E concentration in GSDs with CDRM was significantly greater than in non-GSDs without CDRM. df degrees of freedom, ss sum of squares, ms mean square, F test statistic and p significance level

Dog	Mean	Stdev	Count
1	51.00	*	1
2	76.30	42.00	2
3	45.10	*	1
4	42.00	26.00	2
5	31.25	1.34	2
6	48.30	*	1
7	116.80	33.00	3
8	41.30	4.38	2
9	31.60	7.21	3
10	36.90	*	1
11	36.70	2.26	2
12	29.10	*	1
13	47.10	11.88	2
14	99.80	*	1
15	59.20	*	1
16	46.20	0.99	2
17	46.30	*	1
18	33.50	*	1
19	45.20	*	1
20	33.50	*	1
21	56.30	*	1
22	20.50	*	1
23	63.10	21.80	3
24	83.80	34.80	4
25	37.60	5.80	2

Table 19a. Mean and standard deviation of vitamin E concentrations ($\mu moll^{-1}$) for GSDs with CDRM.

Source	df	ss	ms	F	p	ems
Dog	24	26385	1099	1.96	0.078	$\sigma^2 + \lambda\sigma_D^2$
Error	17	9521	560			σ^2
Total	41	35906				

Using the information from the one-way ANOVA,

$$\lambda = \frac{1}{24}\left(42 - \frac{1}{42}\left\{1^2 + 2^2 + 1^2 + \ldots + 3^2 + 4^2 + 2^2\right\}\right) = 1.663$$

$$\hat{\sigma}^2 = 560$$

$$\hat{\sigma}_D^2 = \frac{1}{1.663}(1099 - 560) = 324$$

Therefore,

Estimate of standard deviation for within dog measurements	23.7
Estimate of standard deviation for between dog measurements	18.0

Table 19b. Results of analysis of variance, and estimates of components of variance for within and between dog measurements for GSDs with CDRM.

Dog	Mean	Stdev	Count
1	24.85	6.29	2
2	17.45	3.32	2
3	29.70	*	1
4	52.60	*	1
5	39.05	13.36	2
6	60.60	*	1
7	38.10	*	1
8	48.60	*	1
9	47.50	*	1
10	23.20	*	1
11	22.60	*	1
12	26.30	*	1
13	44.40	*	1
14	50.50	*	1
15	32.40	*	1
16	47.50	*	1
17	51.45	6.01	2
18	25.45	9.26	2
19	45.95	7.14	2
20	39.15	3.04	2

Table 20a. Mean and standard deviation of vitamin E concentrations ($\mu moll^{-1}$) for GSDs without CDRM.

Source	df	ss	ms	F	p	ems
Dog	19	3897	205	3.49	0.049	$\sigma^2 + \lambda\sigma_D^2$
Error	7	411	59			σ^2
Total	26	4309				

Using the information from the one-way ANOVA,

$$\lambda = \frac{1}{19}\left(27 - \frac{1}{27}\left\{2^2 + 2^2 + 1^2 + \ldots + 2^2 + 2^2 + 2^2\right\}\right) = 1.341$$

$$\hat{\sigma}^2 = 59$$

$$\hat{\sigma}_D^2 = \frac{1}{1.341}(205 - 59) = 109$$

Therefore,

Estimate of standard deviation for within dog measurements 7.7

Estimate of standard deviation for between dog measurements 10.4

Table 20b. Results of analysis of variance, and estimates of components of variance for within and between dog measurements for GSDs without CDRM.

Dog	Mean	Stdev	Count
1	29.00	*	1
2	36.23	4.81	4
3	39.50	4.78	5
4	33.83	5.65	4
5	74.20	*	1
6	42.70	7.21	2
7	33.30	*	1
8	25.93	6.41	3
9	27.20	*	1
10	31.17	7.98	4
11	28.10	*	1
12	26.60	*	1
13	40.16	13.08	5
14	31.72	5.68	4
15	26.92	8.76	4
16	33.80	1.35	3
17	37.10	*	1
18	32.88	3.78	5
19	26.10	*	1
20	28.70	*	1
21	14.00	*	1
22	32.90	*	1
23	45.00	*	1
24	6.68	*	1
25	46.20	4.92	4
26	19.35	5.66	4

Table 21a. Mean and standard deviation of Vitamin E concentrations ($\mu moll^{-1}$) for non-GSDs without CDRM.

Source	df	ss	ms	F	p	ems
Dog	25	5544	222	4.62	0.000	$\sigma^2 + \lambda\sigma_D^2$
Error	38	1822	48			σ^2
Total	63	7366				

Using the information from the one-way ANOVA,

$$\lambda = \frac{1}{25}\left(64 - \frac{1}{64}\left\{1^2 + 4^2 + 5^2 + \ldots + 1^2 + 4^2 + 4^2\right\}\right) = 2.421$$

$$\hat{\sigma}^2 = 48$$

$$\hat{\sigma_D}^2 = \frac{1}{2.421}(222 - 48) = 72$$

Therefore,

Estimate of standard deviation for within dog measurements	6.9
Estimate of standard deviation for between dog measurements	8.5

Table 21b. Results of analysis of variance, and estimates of components of variance for within and between dog measurements for non-GSDs without CDRM.

Group	Within dog variance	df	Between dog variance	df
GSDs with CDRM	560	17	324	24
GSDs without CDRM	59	7	109	19
Non-GSDs without CDRM	48	38	72	25

Table 22. Summary of estimates of within and between dog variances for GSDs with CDRM, GSDs without CDRM and non-GSDs without CDRM.

Variance within GSDs with CDRM: 560

Variance within GSDs without CDRM: 59

$F = 560/59 = 9.49$ $df_1 = 17, df_2 = 7$

$\therefore$ $p = 0.003$

Table 23. Comparison of within dog variances between GSDs with CDRM and GSDs without CDRM.

Variance between GSDs with CDRM: 324

Variance between GSDs without CDRM: 109

$F = 324/109 = 2.97$ $df_1 = 24, df_2 = 19$

$\therefore$ $p = 0.009$

Table 24. Comparison of between dog variances between GSDs with CDRM and GSDs without CDRM.

5. An investigation into the pathology associated with CDRM

5.1 Introduction and Aims

The pathology of the spinal cord in CDRM had been reported by a number of authors (Averill, 1973; Griffiths and Duncan, 1975a; Clemmons, 1989; Clemmons, 1992; Barclay and Haines, 1994). Most descriptions had concentrated on the most severely affected areas of the spinal cord - the mid to caudal thoracic and lumbar segments. Wallerian-type (axonal) degeneration was prominent in the lateral funiculus in the region of the corticospinal and rubrospinal tracts. In addition, more scattered degenerating fibres were present in the remainder of the lateral and ventral funiculi. The dorsal columns also showed changes in both the lumbar and cervical segments. A number of cases exhibited gliosis in the intermediate grey matter of the lumbar enlargement. A smaller number of cases had degeneration of fibres in the lumbar dorsal nerve roots. LMNs appeared to be spared. No specific abnormalities had been reported in the brain, although Clemmons (1989) referred to white matter changes which extended into the brain. The literature on the pathology of CDRM is reviewed in 1 Introduction, page 1.

The purpose of this study was to re-examine the distribution and nature of the spinal cord pathology using classical techniques, electron microscopy and immunocytochemistry. In addition to confirming, or otherwise, the previous reports it was hoped that fresh clues to the pathogenesis and aetiology might be derived from the more modern techniques. A second purpose was to examine carefully the brains of affected dogs to find any previously unreported changes. A detailed analysis of the neuropathology would allow a comparison with neurodegenerative diseases in man for which an aetiology had been determined.

5.2 Materials and Methods

5.2.1 Animals

Twenty GSDs (13 males and seven females) and five GSD crosses (two males and three females) with a clinical diagnosis of CDRM were available for post mortem examination (for details see 2.2 Case Selection, page 32). The dogs were aged seven to 12 years. No significant clinical signs were noted, other than those referable to the neurological disorder. Six dogs, either GSDs or similar sized breeds with no neurological disease were used as one control group. In addition, 11 dogs with defined localised injuries, to the spinal cord were examined as a further control population, to determine the extent of secondary degeneration or retrograde reactions in brain nuclei and tracts following damage to spinal cord axons. Post

mortem examination was performed within two hours of euthanasia which was carried out by intravenous injection of pentobarbitone sodium (Euthatal) (Rhone Merieux Ltd.). The brain and spinal cord were removed while maintaining the continuity of the dural sheath wherever possible. On exposure of the vertebral canal both the dorsal and ventral aspects were examined for evidence of any lesions which might have caused spinal cord compression. Dogs which had either gross evidence or histopathological evidence of cord compression were not included as CDRM cases. SpG were dissected out and removed with the cord. Care was taken to maintain the cord as straight as possible throughout removal from the vertebral canal. The spinal cord was suspended in a long cylinder in 4% BNF (page 202) and weighted to avoid any bending which could have affected the structure. The brain was fixed in 4% BNF with one transverse cut at the level of the optic chiasma to ensure that proper fixation of deep structures occurred.

Details of the CDRM cases used in the pathology study are given in Table 25 (page 115) and Table 26 (page 116). Signalments for dogs in the control group with no neurological lesions dogs are given in Table 27, page 117. Details of the dogs in the control groups with focal spinal cord lesions are given in Table 28 (page 118), Table 29 (page 118) and Table 30 (page 119).

A number of body tissues were collected into BNF from ten of the affected dogs at post mortem, these are detailed in 2.2.3 Collection of samples, page 47.

Three dogs were fixed by intravascular perfusion, after euthanasia, with Karnovsky`s modified fixative (paraformaldehyde/glutaraldehyde 4%/5%). Details of the fixative are given page 202, details of the perfusion technique are given in 2.6.2 Intra-aortic perfusion, page 49. These dogs were at different stages in the clinical course of CDRM.

Case number	Dog ID Number*	Breed	Age at post mortem	Sex
129202	1	GSDX	10y2m	FN
126438	2	GSD	12y3m	FN
126496	4	GSD	8y9m	M
125636	5	GSD	7y8m	M
126403	7	GSD	12y1m	F
128951	19	GSD	9y4m	M
132560	41	GSD	8y7m	M
132913	47	GSD	8y7m	M
128291	49	GSDX	8y5m	FN
126541	55	GSDX	9y6m	F
124705	144	GSD	7y1m	M
CDRM1	146	GSD	10y	M
CDRM2	147	GSD	10y9m	M
124625	160	GSD	10y6m	FN
125570	163	GSD	9y1m	MN
126131	164	GSD	7y1m	M
127150	167	GSDX	10y10m	MN
129481	180	GSD	9y	F

Table 25. Details of CDRM cases for which routine pathology was completed on spinal cord and brain. (* see Table 1, page 33-44).

Case number	Dog ID Number*	Breed	Age	Sex
126586	3	GSD	8y3m	FN
129769	11	GSD	10y3m	F
129800	14	GSD	9y3m	M
129966	31	GSD	9y4m	M
130541	33	GSD	8y7m	F
129057	50	GSDX	12y7m	M
131167	129	GSD	7y	M

Table 26. Details of CDRM cases for which routine pathology was completed on the spinal cord. (* see Table 1, page 33-44)

Dog ID Number*	Sex	Breed	Age (years)	Reason for euthanasia
182	M	GSD	3	Anal furunculosis
183	F	BMD	6.5	Transitional cell carcinoma
184	M	Doberman	9	Colorectal neoplasia
185	FN	Flat coat retriever	8	Malignant histiocytosis
186+	M	Labrador Retriever	9	Mast cell tumour
187	M	GSD	5.5	Testicular neoplasia

+There was no spinal cord pathology completed for this dog.

Table 27. Dogs with no neurological abnormalities. (* see Table 1, pages 33-44)

Dog ID Number*	Sex	Breed	Age (years)	Site/Nature of spinal cord lesion	Age of lesion at post mortem
169	FN	Dachshund	7	T 12/13 Hansen type I disc extrusion	5 days
170	M	Labrador	7	T3 spinal cord compression due to a ventral bony mass	1 week
171	F	Anatolian karabash	8	Brachial nerve root avulsion	7 days
172	F	Great Dane	0.58	Cervical Spondylomyelo-pathy C3, C4, C6/7	2 weeks

Table 28. Dogs with a focal spinal cord lesion, pathology completed on brain and spinal cord. (* see Table 1, pages 33-44).

Dog ID Number*	Sex	Breed	Age (years)	Site/Nature of spinal cord lesion	Age of lesion at post mortem
118	FN	crossbreed	11	T13 disc extrusion	4 days
173	M	Scottish terrier	2	Fibro-cartilaginous embolism (C2/3)	3 days

Table 29. Dogs with a focal spinal cord lesion, pathology completed on spinal cord only. (* see Table 1, pages 33-44)

Dog ID Number*	Sex	Breed	Age (years)	Site/Nature of spinal cord lesion	Age of lesion at post mortem
174	M	Border terrier	6	T1/2 Fibrocartilaginous embolism	4 days
175	M	English pointer	5	C6/7 traumatic disc extrusion	3 weeks
176	M	Labrador	9.5	T3/4 Schwannoma	3 weeks
177	M	Border collie	5	T13/L1 disc prolapse	2 days
178	F	Great Dane	0.66	Cervical spondylomyelopathy C2/3-C6/7	9 days
179	FN	DobX	7	T12/13 disc prolapse	2 weeks
111	M	German pointer	4.5	T7 meningioma	2 weeks

Table 30. Dogs with a focal neurological lesion, pathology completed on brain only. (*see Table 1, pages 33-44)

5.2.2 Processing of tissue for paraffin wax sections

A one cm length of every second spinal cord segment was removed and processed in a Shandon Elliot 24 hour automatic tissue processor (Histokinette) as described in 8.1.2.2 Paraffin wax processing, page 204. A one cm^2 piece of each of the body tissues listed below was removed and processed in the same automatic processor.

The brain was cut into transverse slices. All slices caudal to the level of the optic chiasma were processed in the Shandon Elliot seven day automatic tissue processor (Histokinette) (for details 8.1.2.2 Paraffin wax processing, page 204).

Paraffin-embedded tissue sections were cut on a Biocut 2035 microtome (Leica) and mounted onto APES-coated microscope slides (for details see 2.7.3.2 Preparation of APES coated slides, page 50).

Paraffin wax sections were routinely stained with Haematoxylin and eosin (H&E) and Cresyl violet (CV) (for details see 2.7.4.1.1 Routine stains, page 50).

Paraffin wax sections of the red nucleus from affected and control dogs were stained with Congo Red (for details see 2.7.4.1.1.4 Congo Red, page 51).

5.2.3 Processing of tissue for resin embedding

5.2.3.1 Spinal cord

At the time of post mortem a 2mm slice of one cervical, one thoracic and one lumbar spinal cord segment was taken, cut into quarters and immersed in Karnovsky`s modified fixative (paraformaldehyde/glutaraldehyde 4%/5%) (page 202). These samples were then processed using a Lynx *el* microscopy tissue processor (Leica) for araldite resin embedding (8.1.2.1 Resin processing, page 203).

5.2.3.2 Brain

Red nucleus tissue for resin embedding was collected from cases perfusion-fixed with Karnovsky`s modified fixative (paraformaldehyde/glutaraldehyde 4%/5%) (as above). On removal from the perfused dog, the brain was immersed in the same fixative until complete fixation had occurred. The red nucleus was dissected out and sliced at 2mm, then processed using a Lynx *el* microscopy tissue processor (Leica) for araldite embedding (as above).

5.2.4 Immunocytochemistry

A number of antisera were used in an attempt to define more accurately the nature of the lesions. Dogs with identified focal spinal cord lesions were investigated in parallel, to determine if the changes occurring in the CDRM dogs were specific to the disease.

Immunostaining was carried out by three basic techniques; (i) the PAP technique; (ii) the ABC technique which was similar, but employed a biotinylated secondary antibody and a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (iii) double indirect immunofluorescence. These techniques are all described in detail in 2.7.4.1.2 Immunocytochemistry, page 51. Complete lists of the antisera used for immunostaining are given in Table 3 (page 54, PAP techniques), Table 4 (page 55, ABC technique) and Table 5 (page 56, immunofluorescent techniques).

The PAP technique was employed for SMI 31, RT-97, RM017, RM024 using mouse PAP complex (Sigma) at 1/1250 and a goat α-mouse link Sigma) at 1/10. The PAP technique was also used for α-GFAP, α-PLP 226, Affiniti NA 1297, α-ubiquitin and α-MBP. This group used a rabbit PAP complex (ICN Immunobiologicals) at 1/40 and a goat α-rabbit link (Sigma) at 1/10.

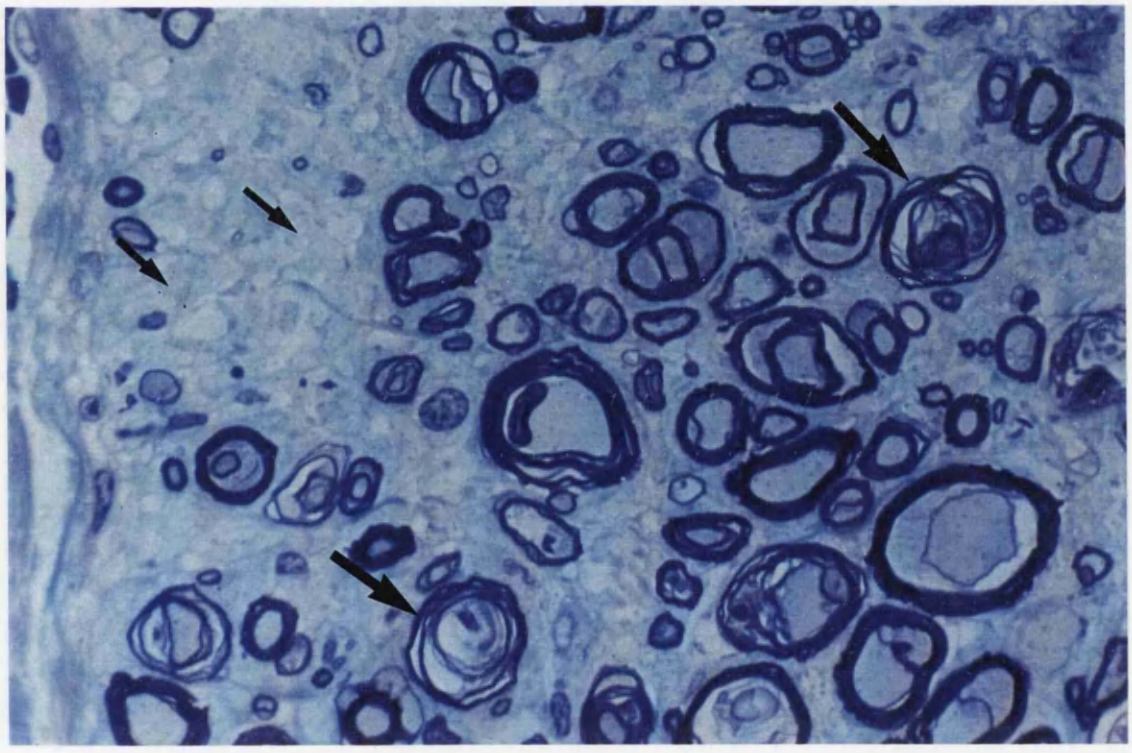
5.3 Results

5.3.1 Histology of spinal cord of dogs with CDRM

5.3.2 White Matter

Pathological changes in the white matter involved axonal degeneration with a concomitant loss of myelin in affected areas, often with an accompanying gliosis. The glial reaction consisted predominantly of microglia, with some increase in astrocytes (Figure 6, page 122). The allocation of cell types, in sections stained with H&E, was based on the nuclear appearance. Microglia have an elongated, rod-like nucleus, astrocytes have a more rounded, paler nucleus due to less chromatin. Some degenerating fibres had associated macrophages. The patterns of degeneration are shown in Figure 7, page 123-125.

(a)



(b)

Figure 6. Resin sections (1μm) from a dog with CDRM (a) dorsal columns showing loss of axons and myelin sheaths in the fasciculus gracilis (b) the dorsolateral column at higher magnification to show fibre degeneration (large arrows) and the presence of astrocyte processes (small arrows). (a)x150(b)x1000 (a) and (b)C4

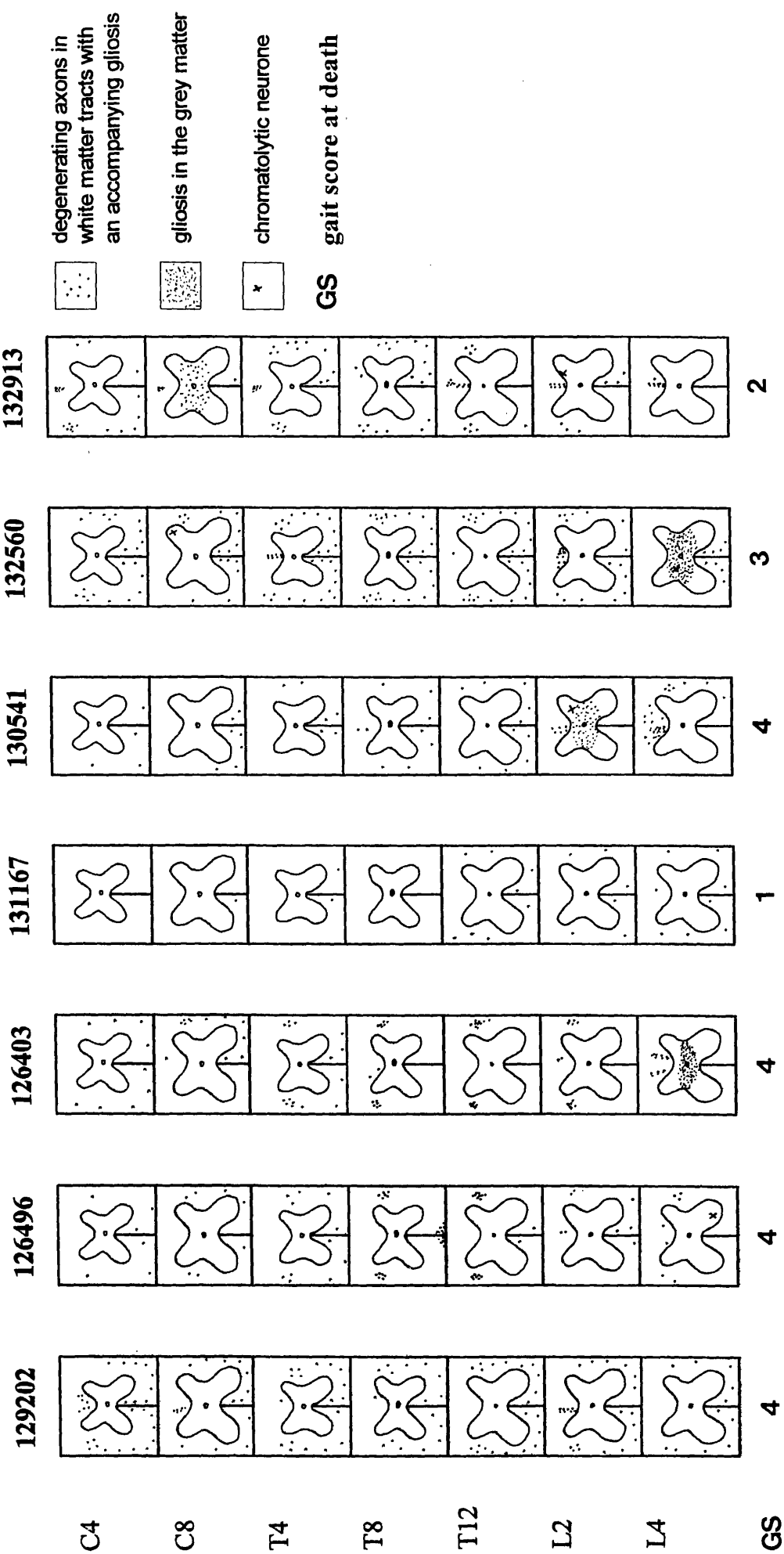


Figure 7a. Patterns of spinal cord degeneration found in dogs with CDRM.

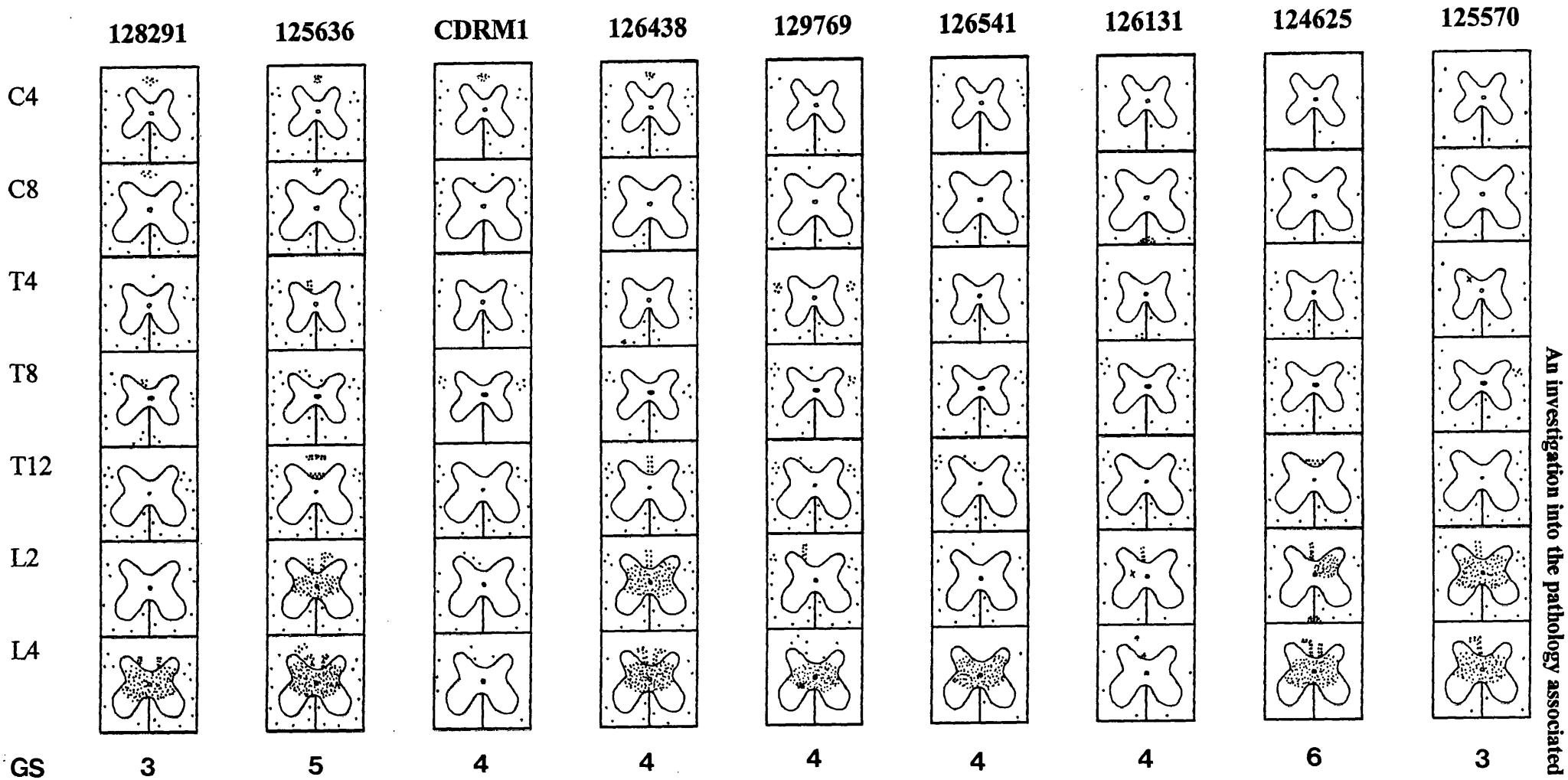


Figure 7b. Patterns of spinal cord degeneration found in dogs with CDRM. (For key see Figure 7a, page 119).

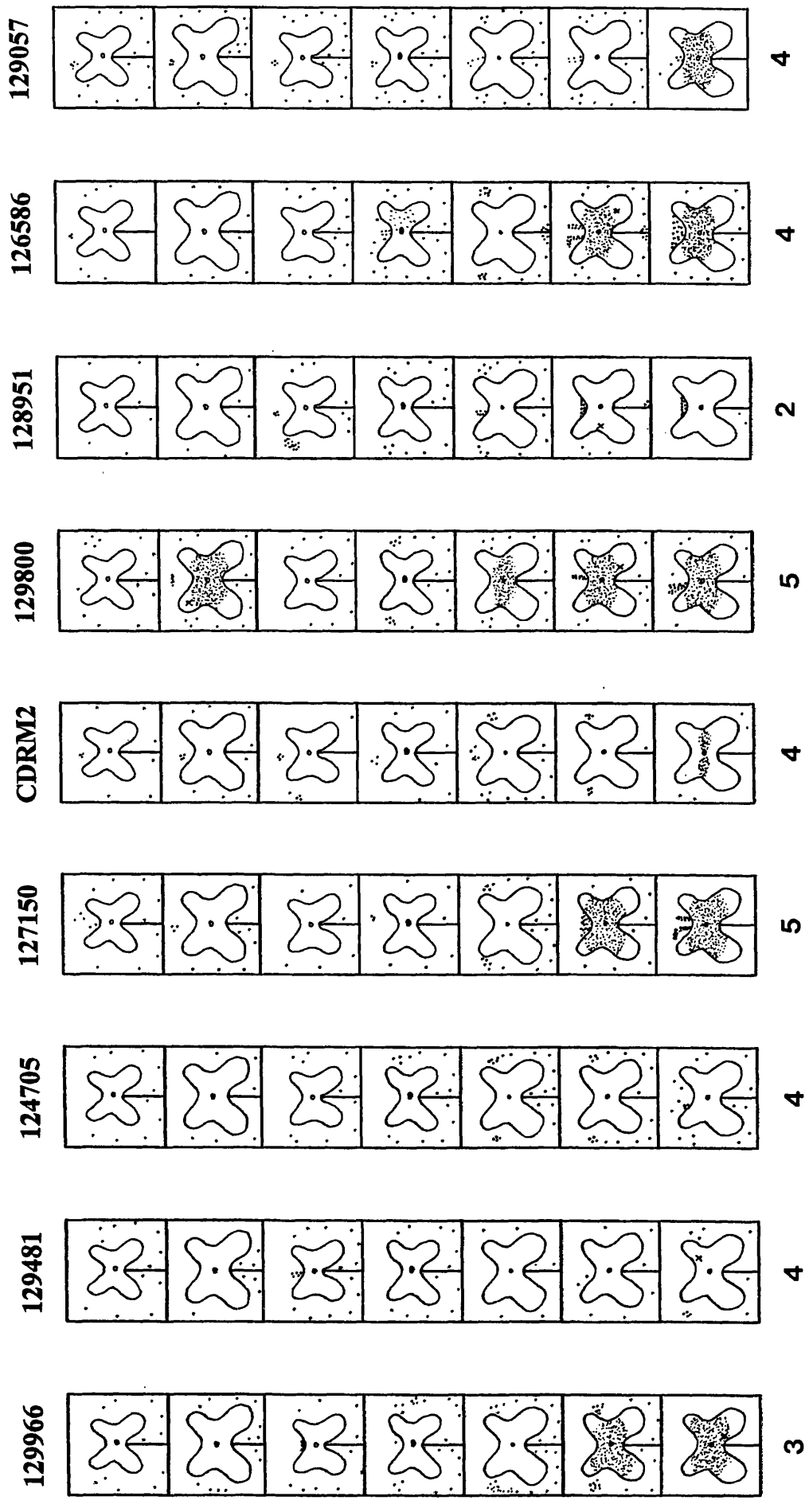


Figure 7c. Patterns of spinal cord degeneration found in dogs with CDRM. (For key see Figure 7a, page 119).

5.3.2.1 Dorsal funiculus

White matter changes in the dorsal funiculus varied widely from dog to dog both in severity and distribution. The severity ranged from occasional scattered fibre degeneration to areas of fibre loss with an associated gliosis. These changes were found exclusively in the fasciculus gracilis. Dogs could be grouped according to the distribution of degeneration through the length of the dorsal funiculi. The two most common patterns were changes throughout cervical, thoracic and lumbar regions of the spinal cord, although not necessarily in every segment in each case (9/25), or degeneration in the lumbar segments only (5/25). Other variations were involvement of the cervical and lumbar segments with sparing of the thoracic cord (3/25); thoracic and lumbar involvement only (5/25); thoracic segments only (1/25) and finally cervical and thoracic regions only (1/25). Only one of the dogs (Dog 55) had no dorsal funiculus changes at all. There were varying degrees of asymmetry seen in the dorsal funiculi at all levels.

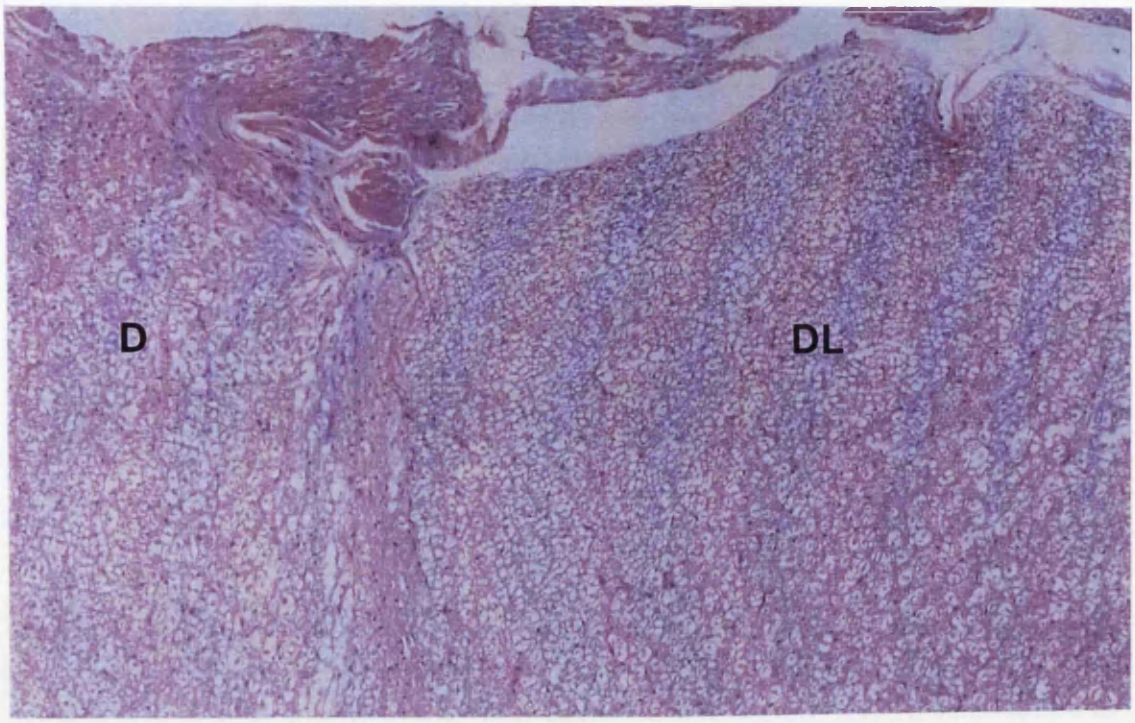
5.3.2.2 Lateral funiculus

The lateral funiculus consistently showed lesions. In all cases, at least a few scattered degenerate fibres were present around the periphery of the cord, not always including the ascending dorsal spinocerebellar tracts. Most of the cases (16/25) had occasional fibre degeneration around the periphery with a greater number of degenerated fibres in the region of the corticospinal and rubrospinal tracts (Figure 8, page 127). All of these had an associated gliosis in the region of the descending tracts.

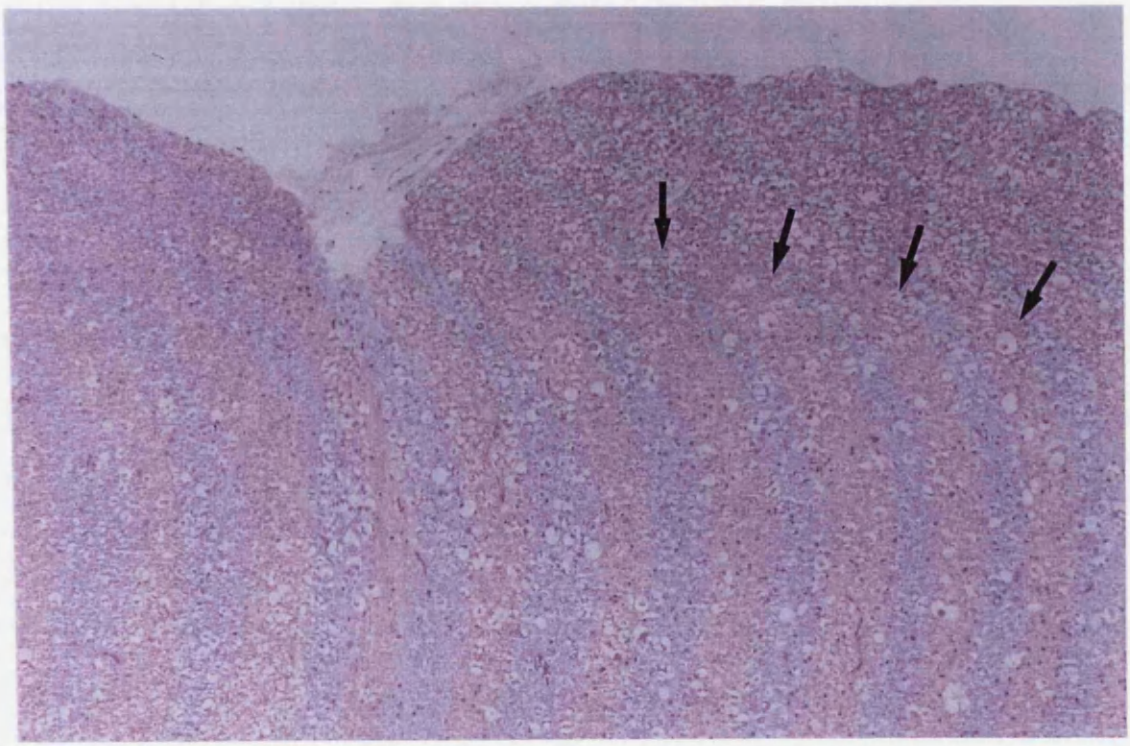
5.3.2.3 Ventral funiculus

Consideration of the ventral funiculus showed that most of the degeneration was in the descending tracts which surrounded the ventromedian fissure. Degeneration tended to involve all the descending tracts in this region, namely the MLF, medial vestibulospinal tract, ventral corticospinal tract and the tectospinal tract. Four cases showed an associated gliosis in the tectospinal tract; this was a localised occurrence in each case and occurred in one spinal cord segment only.

Considering the patterns of degeneration in the lateral and ventral funiculi together, three cases showed degeneration in the regions of the corticorubrospinal tracts and around the ventromedian fissure only, with sparing of the rest of the white matter.



(a)



(b)

Figure 8. Spinal cord segments stained with H&E (a) from a normal dog showing unaffected dorsolateral (DL) column and dorsal column (D) and (b) from a CDRM case to show the degeneration (upper edge outlined by arrows) in the dorsolateral columns with sparing of the dorsal columns. Magnification x105. (a) and (b)C8

5.3.3 Grey Matter

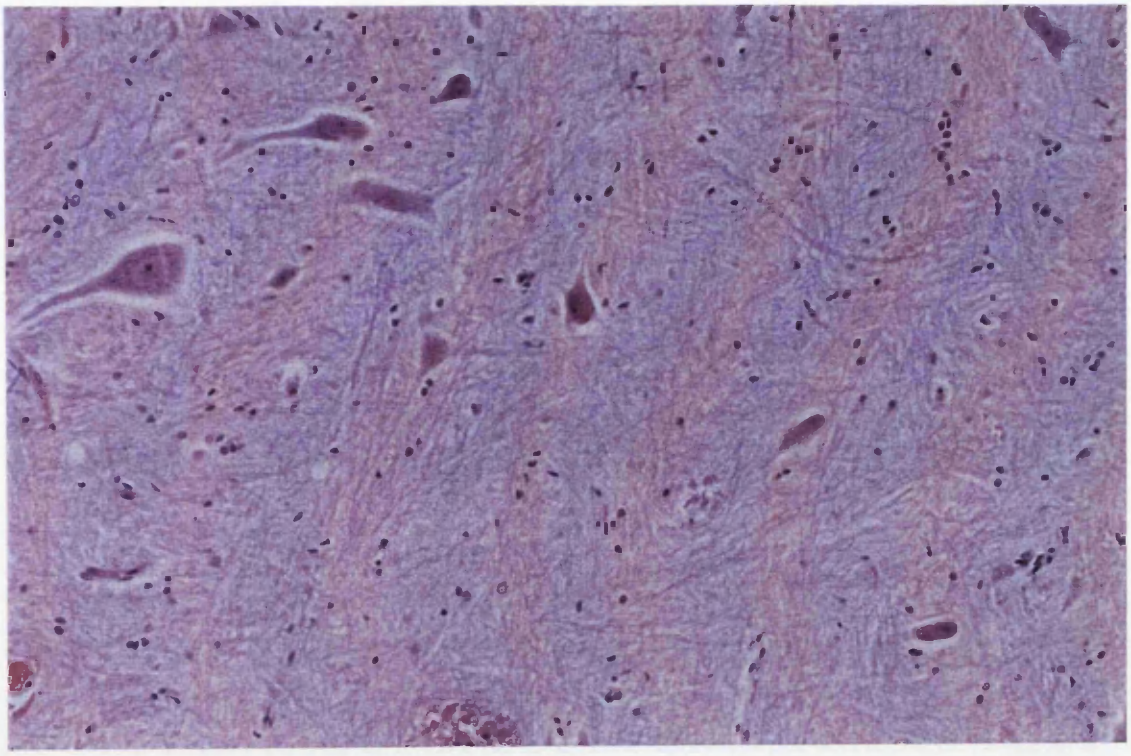
Pathological changes in the grey matter were less consistent. The dogs fell into three groups; those that had no grey matter changes (5/25); those having occasional chromatolytic neurones (9/25); and the largest group, which had gliosis in the grey matter (16/25), often with chromatolytic neurones (6/25). Grey matter gliosis in most cases extended throughout the intermediate horn (Figure 9, page 129). In all but two cases, the gliosis was bilateral. In the first of these two cases (Dog 160) the gliosis was asymmetric in spinal cord segment L2 but was symmetrical by L4. In the second case (Dog 3), spinal cord segment T8 showed a slight gliosis in the right horn of the grey matter, which was symmetrical by L2. As a general rule, the grey matter gliosis occurred only in the lumbar segments. Two cases (Dogs 14 and 47) varied from this and had a slight gliosis in the grey matter of spinal cord segment C8. This was an isolated finding in both, as there was no further gliosis in Dog 14 cranial to T12 and no gliosis in any of the thoracic or lumbar spinal cord segments of Dog 47. Occasional chromatolytic neurones were seen, most commonly in the intermediate grey matter of the lumbar spinal cord segments.

5.3.4 Histology of spinal cord of dogs with focal spinal cord lesions

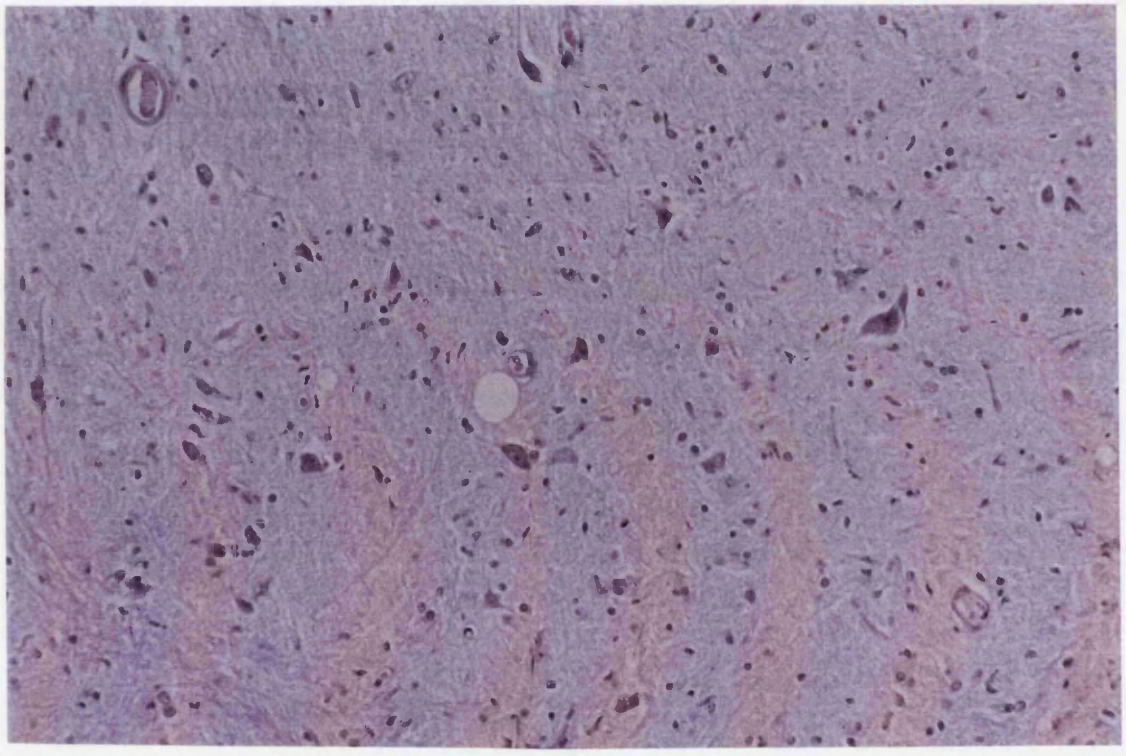
Details of the spinal cords of six dogs with focal spinal cord lesions are given in Table 28 (page 118) and Table 29 (page 118). The first of these was a seven year old Dachshund with a T12/13 disc extrusion of five days duration. Cranial to the lesion there was a mild gliosis in the fasciculi gracili. Caudal to the lesion there were a very small number of degenerating axons around the periphery of the ventral and lateral columns of the white matter. One chromatolytic neurone was found in a SpG from the lumbar enlargement.

The second case was a seven year old Labrador with spinal cord compression at T3 due to the presence of a ventral bony mass. The clinical signs were of seven days duration. Cranial to the lesion the only evidence of degeneration was a mild gliosis in one fasciculus gracilis. Caudal to T3 there was loss of axons around the periphery of the ventral and lateral columns of the white matter, which involved all descending tracts.

The third case was an 11 year old crossbreed with a disc extrusion at T13 of four days duration. Cranial to the lesion, the white matter was normal but the grey matter was atrophied with an associated greatly enlarged central canal. Caudal to the lesion the white matter was again unaffected but there was gliosis in the grey matter. The spinal nerve roots throughout the length of the spinal cord showed vacuolation. The glia limitans appeared thickened throughout the length of the spinal cord.



(a)



(b)

Figure 9. Sections of the lumbar spinal cord grey matter to show density of glial cell population (a) from a normal dog and (b) from a dog with CDRM showing an increased number of glial cells in the intermediate grey matter. Magnification x210. (a) and (b) L2

The fourth case was a seven month old Great Dane with a two week history of signs associated with cervical spondylomyelopathy. The most severely affected region was C6/7. Cranial to the lesion, degenerated axons were found in the fasciculus gracilis and the fasciculus cuneatus. The dorsal spinocerebellar tracts had a mild gliosis. Caudal to the lesion, there were degenerate axons in the area of the corticospinal tracts and in the ventral columns.

The fifth case was a two year old Scottish terrier with fibrocartilaginous embolism of three days duration. Cranial to C6 the only evidence of degeneration was a chromatolytic neurone in the grey matter. Spinal cord segment C6 had many degenerate axons in the lateral columns with complete tract degeneration in the ventral columns and an associated cellular infiltration. The degeneration was much less severe in spinal cord segment C7 and affected only the grey matter and lateral column of one side.

The last case was an eight year old Anatolian karabash with a brachial plexus avulsion of seven days duration. As the injury occurred in the peripheral nervous system it was not exactly comparable to damage which occurs to neurones contained entirely within the CNS. It was included, however, to evaluate retrograde axonal reaction of defined duration. At the level of the cervical enlargement the population of neurones in the ventral horn ipsilateral to the avulsion were all chromatolytic; there was no associated gliosis. Caudal to this lesion the population of ventral horn neurones were normal.

5.3.5 Histology of spinal cord of neurologically normal dogs

Examination of the spinal cords of age-matched dogs with no neurological deficits showed very occasional isolated degenerated fibres with no associated gliosis. The only consistent lesion was an occasional vacuolation of myelin sheaths in the spinal nerve roots, that occurred in the older control dogs.

5.3.6 Histology of brain of dogs with CDRM

Brains from 18 dogs affected by CDRM were examined, these are listed in Table 25, page 115. Pathological changes in the brain were found in both brain nuclei and white matter structures; the changes in the brain nuclei occurred more consistently.

5.3.6.1 Brain nuclei

Affected neurone populations were the red nucleus, the LVN and the lateral (dentate) nucleus. A number of changes were seen: mild gliosis with no associated neurone changes; neurones with eccentric nuclei and chromatolytic neurones with,

or without, an associated gliosis (see Figure 10, page 133). In some severely affected dogs the number of neurones appeared to be reduced compared with the equivalent neuronal population in unaffected dogs suggesting that an actual loss of neurones had occurred.

The red nucleus was affected in all dogs examined and was the most severely affected nucleus. Gliosis was present in each dog. In 17 cases this was uniform throughout the nucleus, although the severity varied between cases. In one case (Dog 144) the gliosis was limited to the dorsolateral portion of the nucleus. Fifteen cases had evidence of chromatolytic neurones, two of these also had neurones with eccentric nuclei. One of the remaining three cases had several neurones with eccentric nuclei. Neurone numbers appeared to be reduced in three of the cases, in comparison with the neurologically normal control group.

The LVN was affected in all cases but the changes were less severe than those in the red nucleus. All cases had evidence of gliosis, the severity varying from mild to severe. Gliosis, if present, was uniform throughout the nucleus. Twelve cases had chromatolytic neurones, and of these, two also had neurones with eccentric nuclei. Only one case had evidence of neuronal loss.

The lateral nucleus was less consistently affected, only eight dogs had evidence of degeneration. Most of these had a mild gliosis only, while two had an occasional chromatolytic neurone. One case had evidence of a neuronal loss.

5.3.6.2 White matter

White matter changes consisted of gliosis of varying severity and occasional degenerate fibres or axonal spheroids. Evidence of degeneration was found in two regions, the ventral tegmental decussation and the tract into the nodulus.

The ventral tegmental decussation had evidence of degeneration in 16/18 cases (Figure 11, page 134). The most common change was gliosis, this varied from mild (12/18) to severe (4/18). Macrophages were seen in association with degenerating fibres in six cases.

The tract into the nodulus was affected in 10/18 cases, of which nine exhibited a mild gliosis and one a severe gliosis. In only one case was an axonal spheroid seen.

5.3.7 Histology of brain of neurologically normal dogs

Brains from six dogs with no neurological abnormalities were examined. The only changes were occasional lipofuscin granules in the neurones of the red nucleus.

5.3.8 Histology of brain of dogs with focal spinal cord lesions of known duration

Brains from 11 dogs with focal spinal cord lesions of known duration were examined. The most prolonged of these had had a three week duration of clinical signs, so these were all of much shorter duration than CDRM. In the majority of cases there was an adequate number of neurones and no evidence of a gliosis (Figure 12, page 135). However, a small number of red nuclei (3/11) showed occasional changes. One of these cases (Dog 175) had chromatolytic neurones in the red nucleus although there was no associated gliosis and no evidence of a loss of neurones. One of the Great Danes with cervical spondylomyelopathy (Dog 172) had one neurone with a slightly eccentric nucleus. The only other case with obvious changes in the red nucleus was the dog with the brachial nerve root avulsion (Dog 171); this dog had two chromatolytic neurones in one red nucleus.

The LVN showed changes in three of the cases. One dog had chromatolytic neurones with no associated gliosis (Dog 169) while two (Dogs 176 and 175) had chromatolytic neurones with an associated gliosis.

The lateral nucleus was unaffected in this group of dogs.

White matter changes were not observed in this group of dogs.

5.3.9 Histology of body tissues from affected dogs

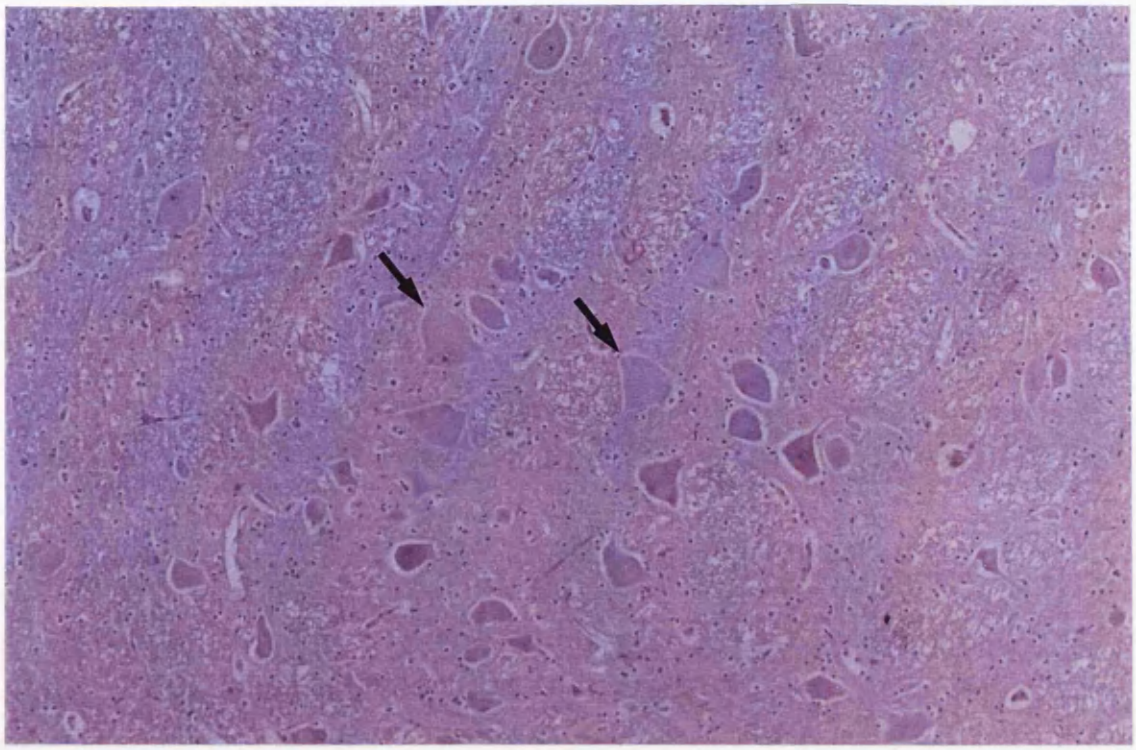
No significant abnormalities were found in any of the body tissues examined.

5.3.10 Histology of the peripheral nervous system

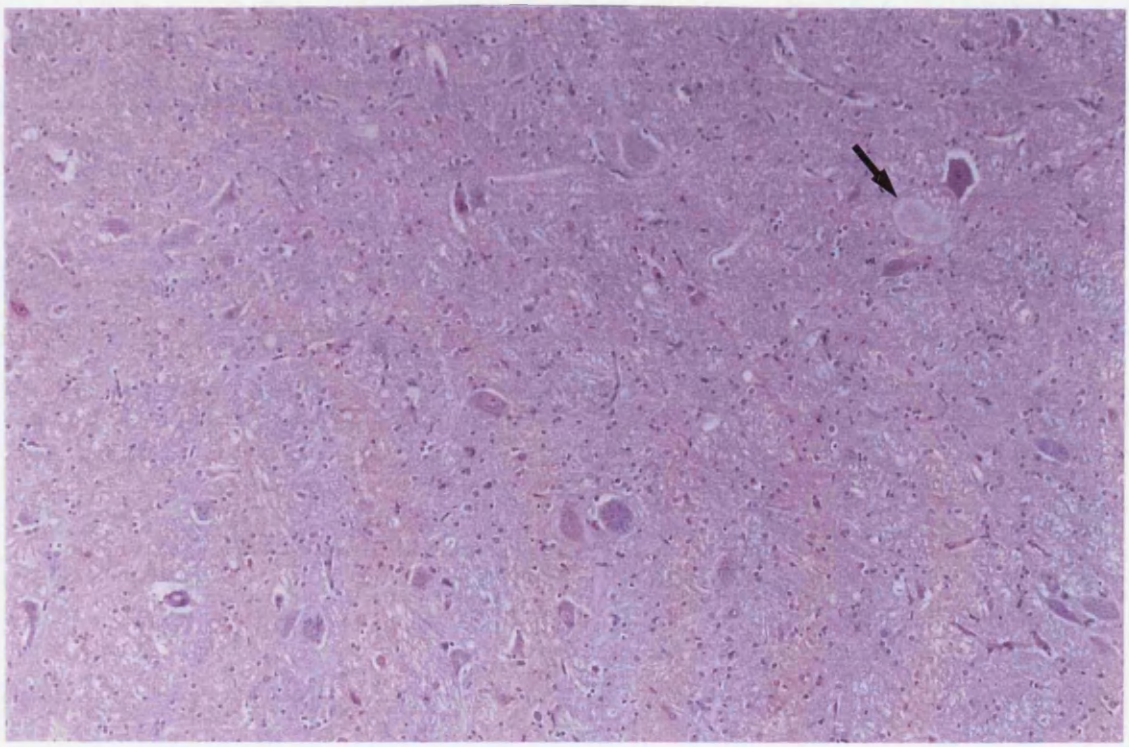
Stellate ganglia, cranial mesenteric ganglia, optic nerve and sciatic nerve showed no abnormalities in any of the affected dogs.

5.3.11 Histology of the spinal ganglia (SpG)

Several of the affected dogs had occasional chromatolytic neurones in the SpG associated with both the cervical and lumbar enlargements. A number of dogs with focal spinal cord lesions also had an occasional chromatolytic neurone in the SpG. A chromatolytic neurone was observed in the SpG of only one of the neurologically normal dogs (Dog 183).



(a)



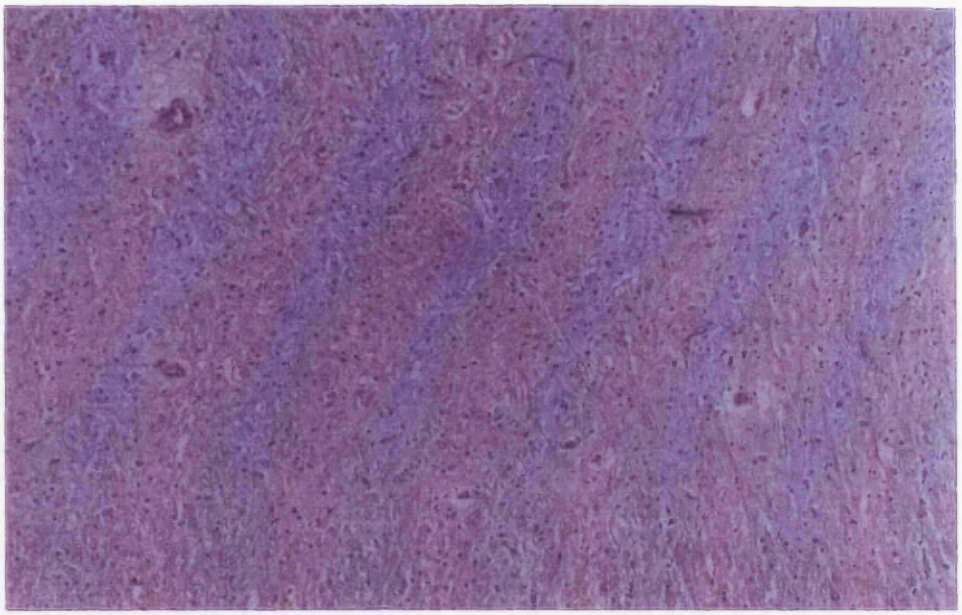
(b)

Figure 10. Sections of red nucleus stained with H&E (a) from a dog with a C6/7 traumatic disc extrusion of 3 weeks duration to show the normal number of neurones. Many neurones are chromatolytic (arrows), some of which have eccentric nuclei. (b) from a dog with CDRM showing a chromatolytic neurone (arrow), a marked reduction in the number of neurones and an increase in glial cell numbers. Magnification x105.

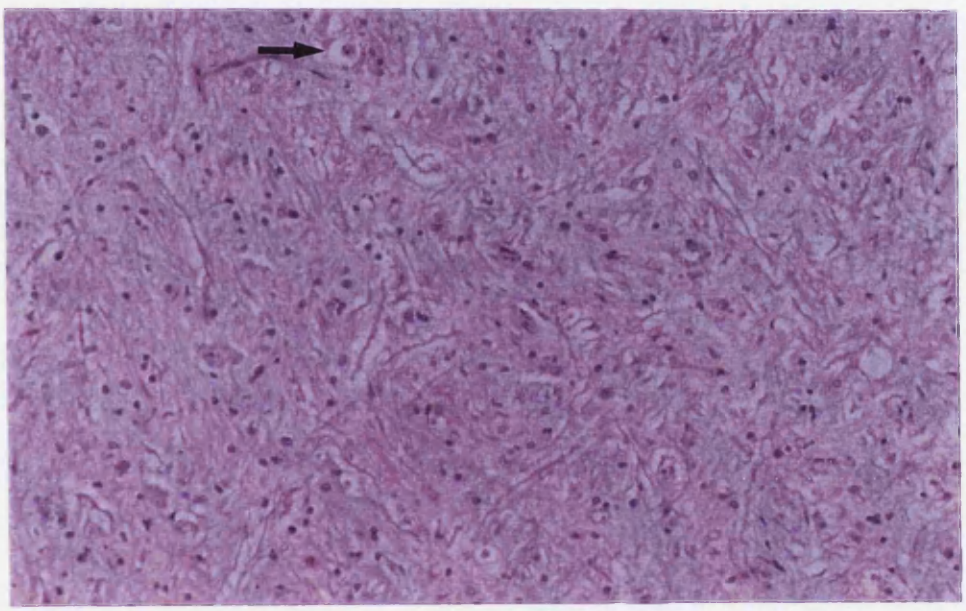
Figure 11. Sections from the ventral tegmental decussation stained with H & E. (a) from a dog with CDRM showing a gliosis. Magnification x95. (b) from the same dog at higher magnification showing a myeloclast (arrow). Magnification x190. (c) from a dog with a C6/7 lesion of three weeks duration. Magnification x95.

Facing page 134

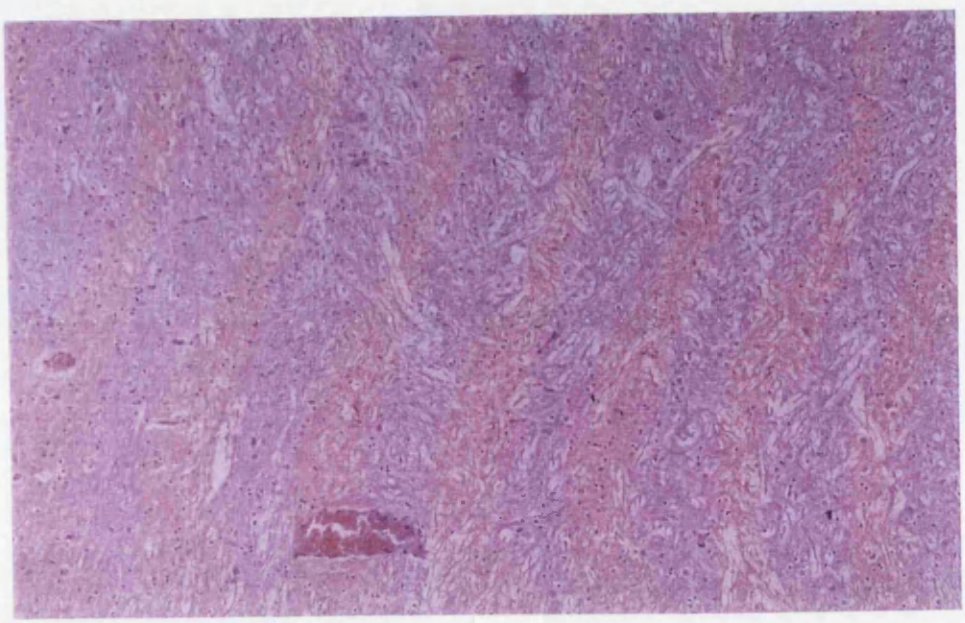
(a)

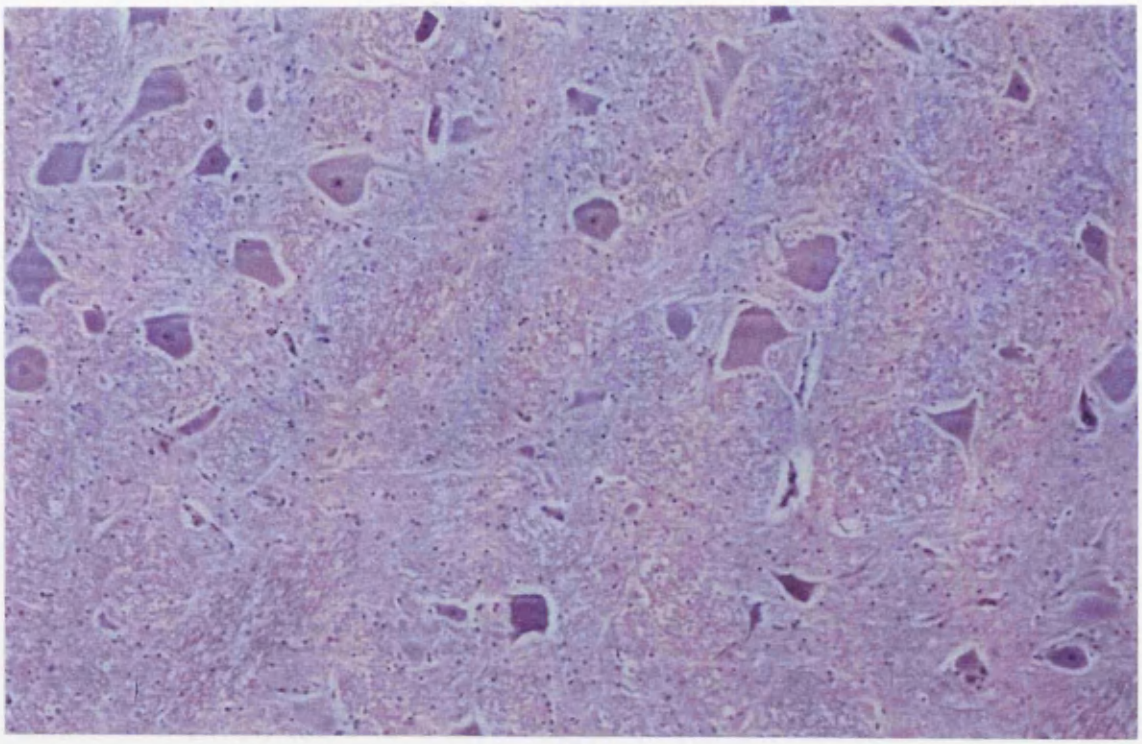


(b)

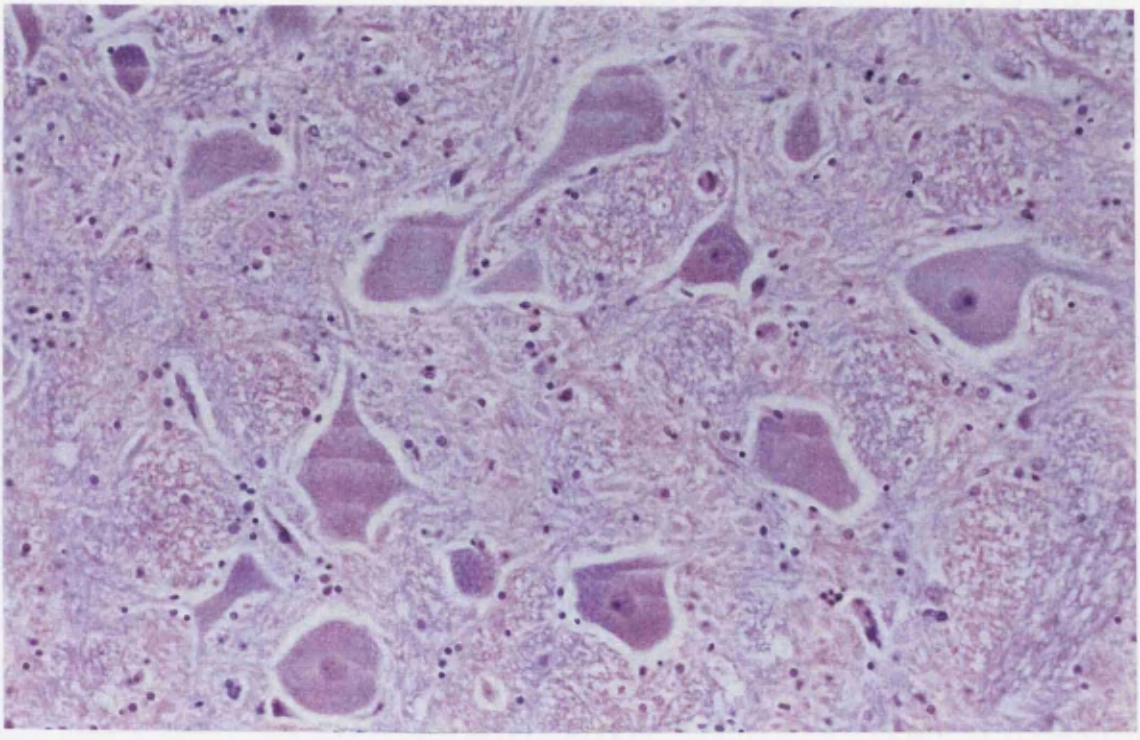


(c)





(a)



(b)

Figure 12. Section of red nucleus from a dog with a spinal cord lesion (T1/2 Fibrocartilaginous embolism) of four days duration (a) at low power to show the relatively high number of neurones compared with CDRM cases. Magnification x105. (b) at higher power to show the absence of a gliosis. Magnification x230.

5.3.12 Ultrastructural analysis of spinal cord and red nucleus of dogs with CDRM

5.3.12.1 Spinal cord

The electron microscopy study of the spinal cord was undertaken to confirm that both the axon and myelin sheath were affected in CDRM and to determine if the early axonal changes might suggest a specific nature for the disease.

Both degenerated axons and myelin sheaths were found in all spinal cord segments examined, within the abnormal areas defined by light microscopy. Astrocyte processes were prominent within the degenerated regions (Figure 13, pages 137-138). Vacuolated macrophages were found in association with a number of the degenerated axons. The appearance was typical of Wallerian-type degeneration.

5.3.12.2 Brain nuclei

Changes were found in the two nuclei examined, the red nucleus and the LVN. The abnormalities were identical and the majority of information was gathered from the red nucleus. Many neurones appeared normal with well defined Nissl bodies and Golgi apparatus; lipofuscin granules were often prominent (Figure 14, pages 139-140). Affected neurones and dendrites showed loss of the Nissl granules and dispersion of the rough endoplasmic reticulum (RER). Ribosomes tended to be present as monosomes or polysomes rather than associated with cisternae of endoplasmic reticulum. Neurofilaments were often increased (Figure 15, pages 141-143). Commonly, enmeshed within the neurofilaments, were collections of mitochondria and vesicles of various sizes, the origin of which were not determined. Occasional myelinated axons were also enlarged by accumulated neurofilaments and/or membranous organelles (Figure 16, pages 144-145).

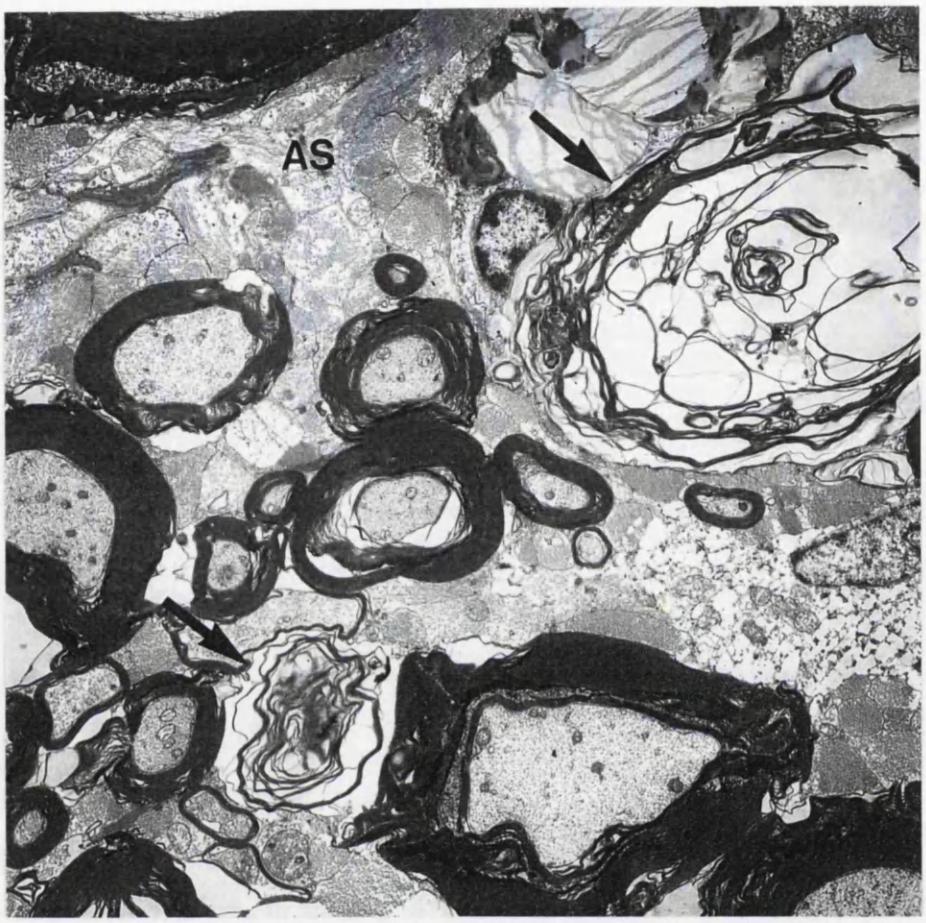


Figure 13a. Electronmicrograph from the dorsal quadrant of spinal cord segment T12 from a dog with CDRM (a) this micrograph shows two degenerate fibres (arrows), but no evidence of demyelinated axons. There is an increased number of astrocyte processes (AS). Magnification approximately x5000.

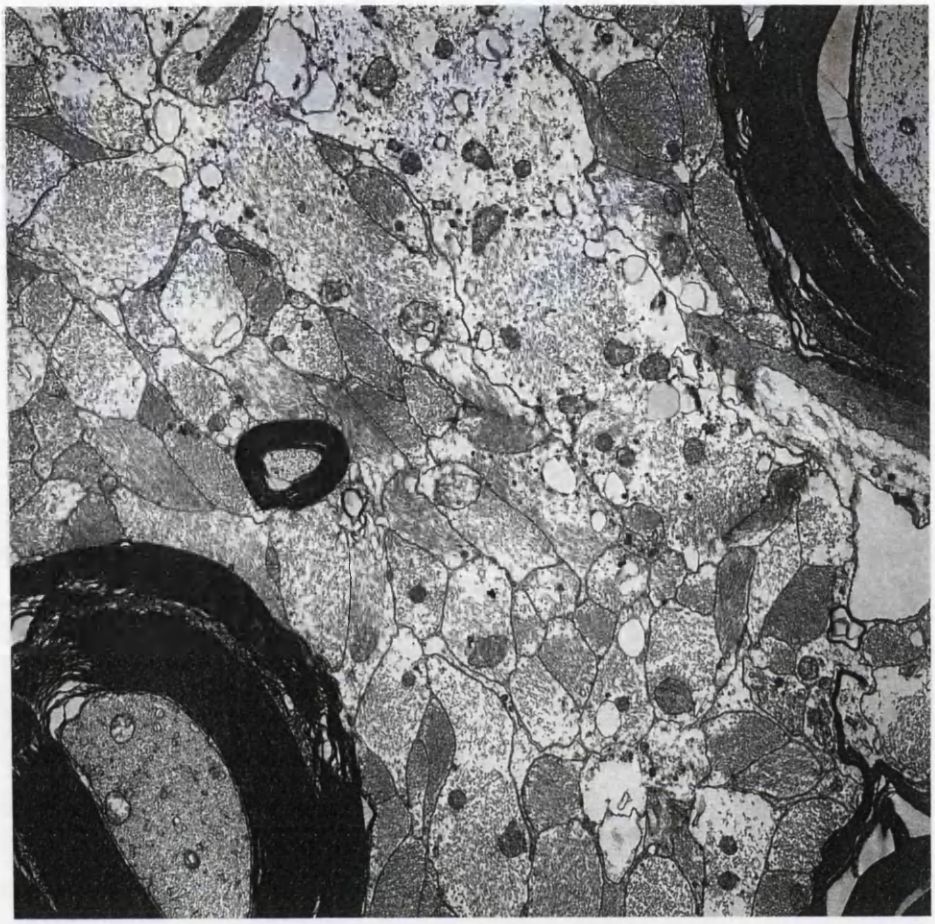


Figure 13b. Electronmicrograph from the dorsal quadrant of spinal cord segment T12 from a dog with CDRM (b) Region of longstanding degeneration as shown by loss of fibres and a marked increase in astrocyte processes. Magnification approximately x8000.

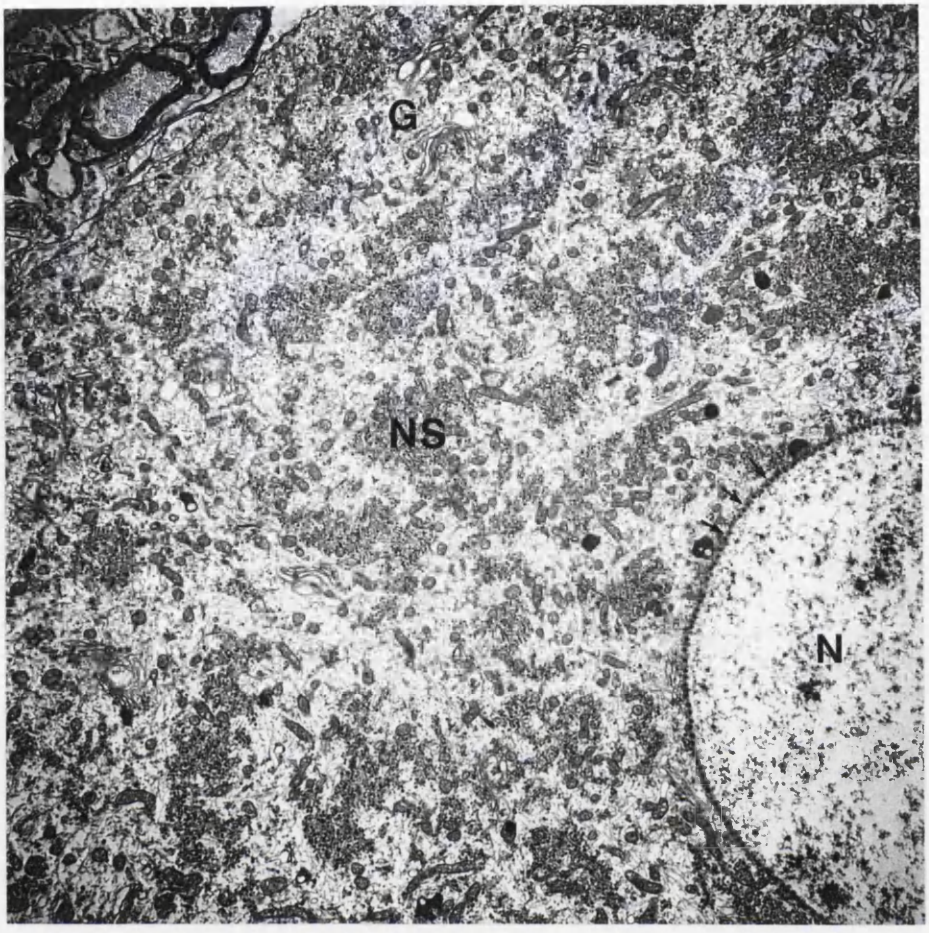


Figure 14a. Electronmicrographs of normal neurones from the red nucleus of a dog with CDRM. (a) The nucleus (N) and associated nuclear pores (small arrows) is present. Nissl granules (NS) are well-defined and interspersed with clearer areas containing neurofilaments, microtubules and Golgi apparatus (G). Magnification approximately x5000.

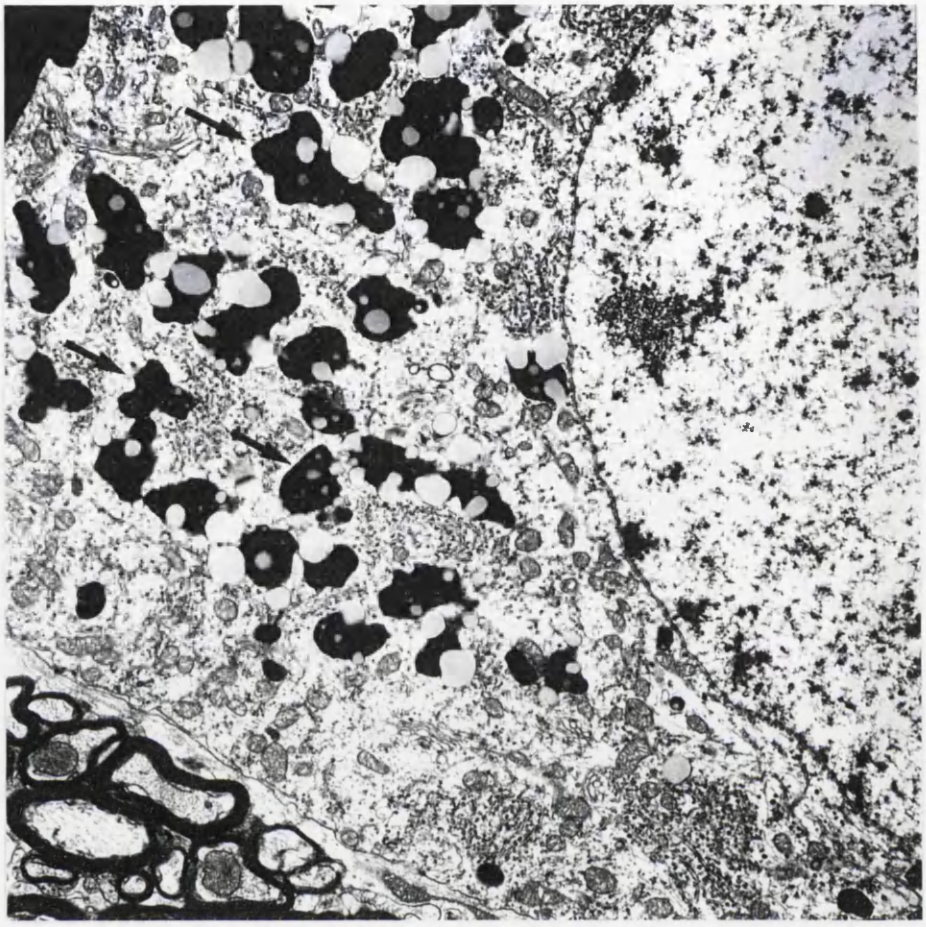


Figure 14b. Electronmicrographs of normal neurones from the red nucleus of a dog with CDRM. (b) This higher magnification shows numerous lipofuscin granules (arrows). Magnification approximately x8000.

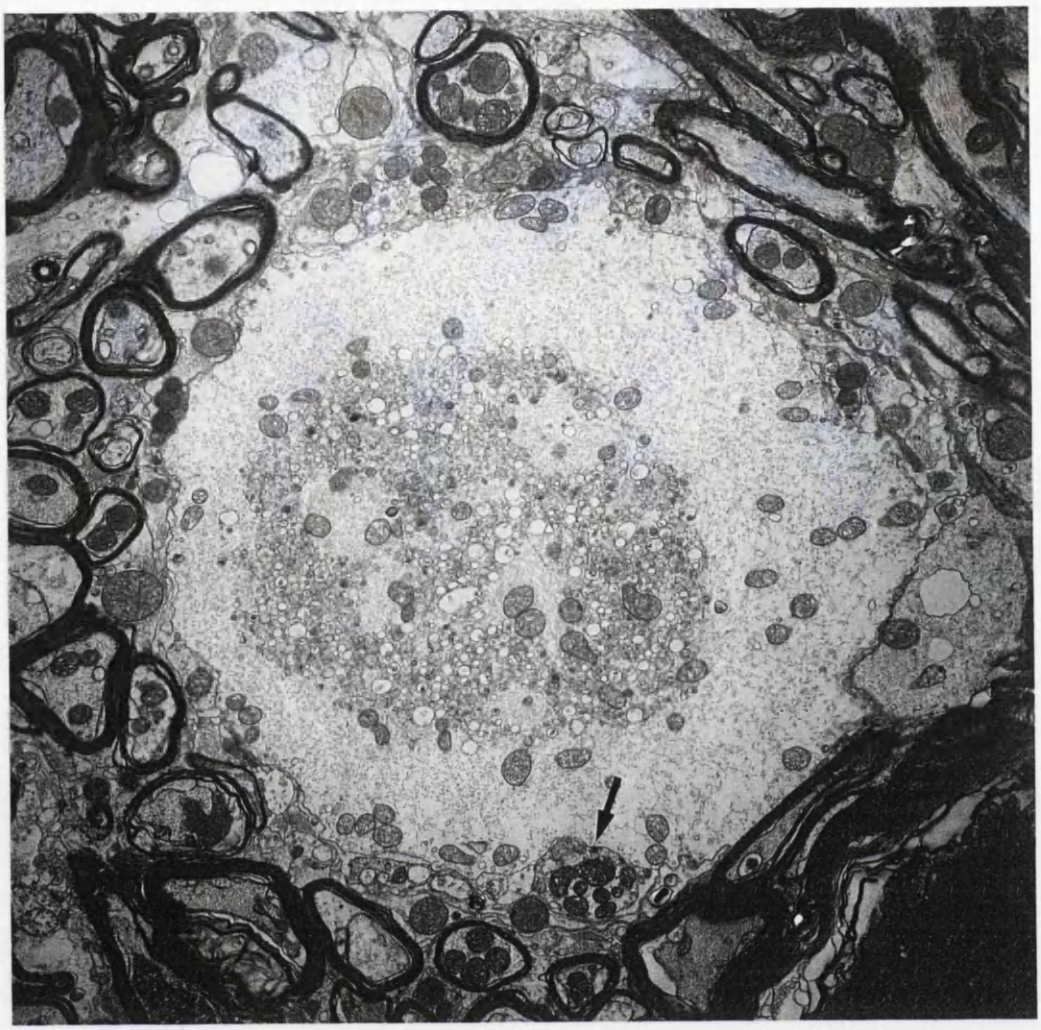


Figure 15a. Electronmicrographs of a neurone from the red nucleus of a dog with CDRM (a) this micrograph shows a dendrite, recognisable due to the presence of synapses (arrow). There is an increase in neurofilaments and accumulation of organelles in the centre of the process. Magnification approximately x6000.

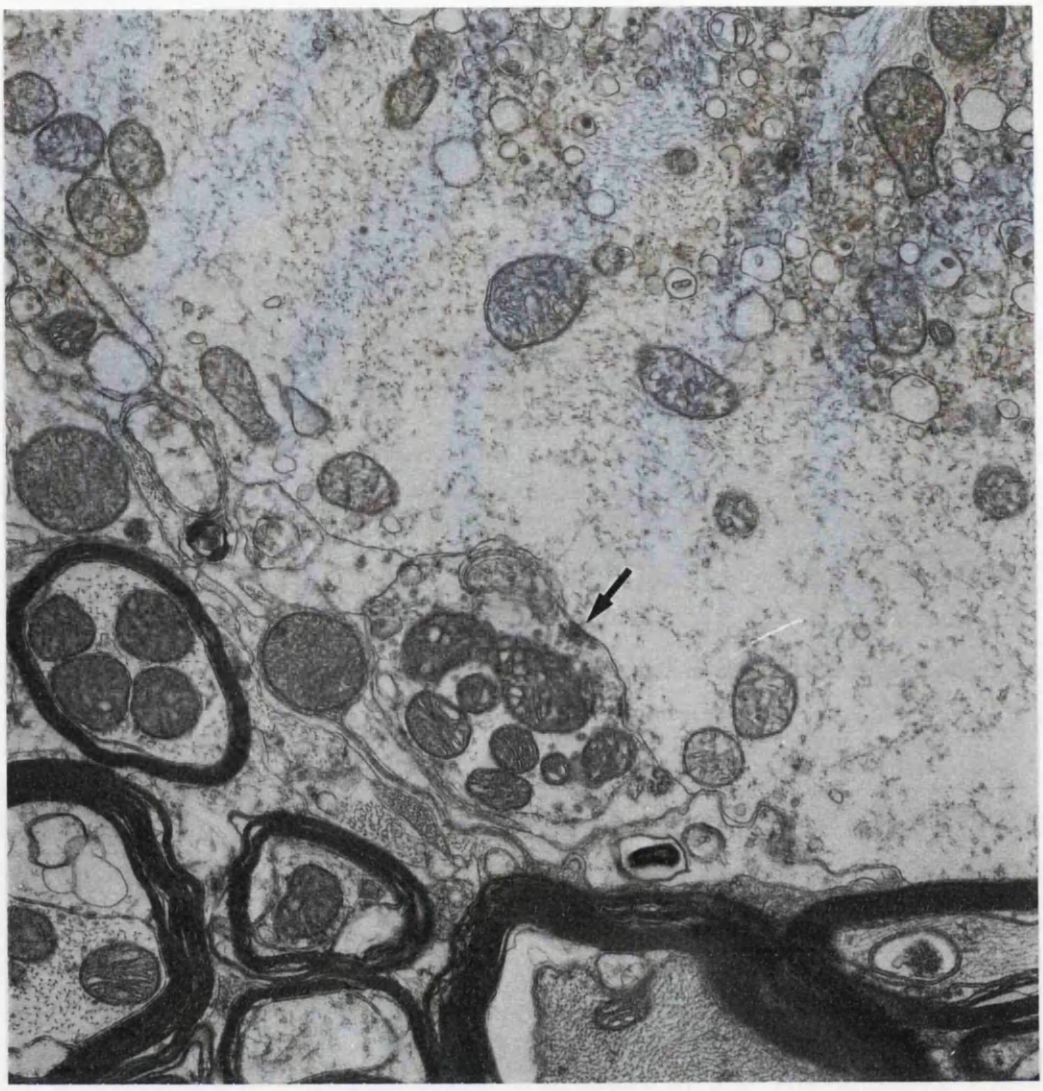


Figure 15b. Electronmicrograph of the neurone from Figure 15 (a) showing the synapse illustrated in Figure (a) (arrow). Magnification approximately x19000.

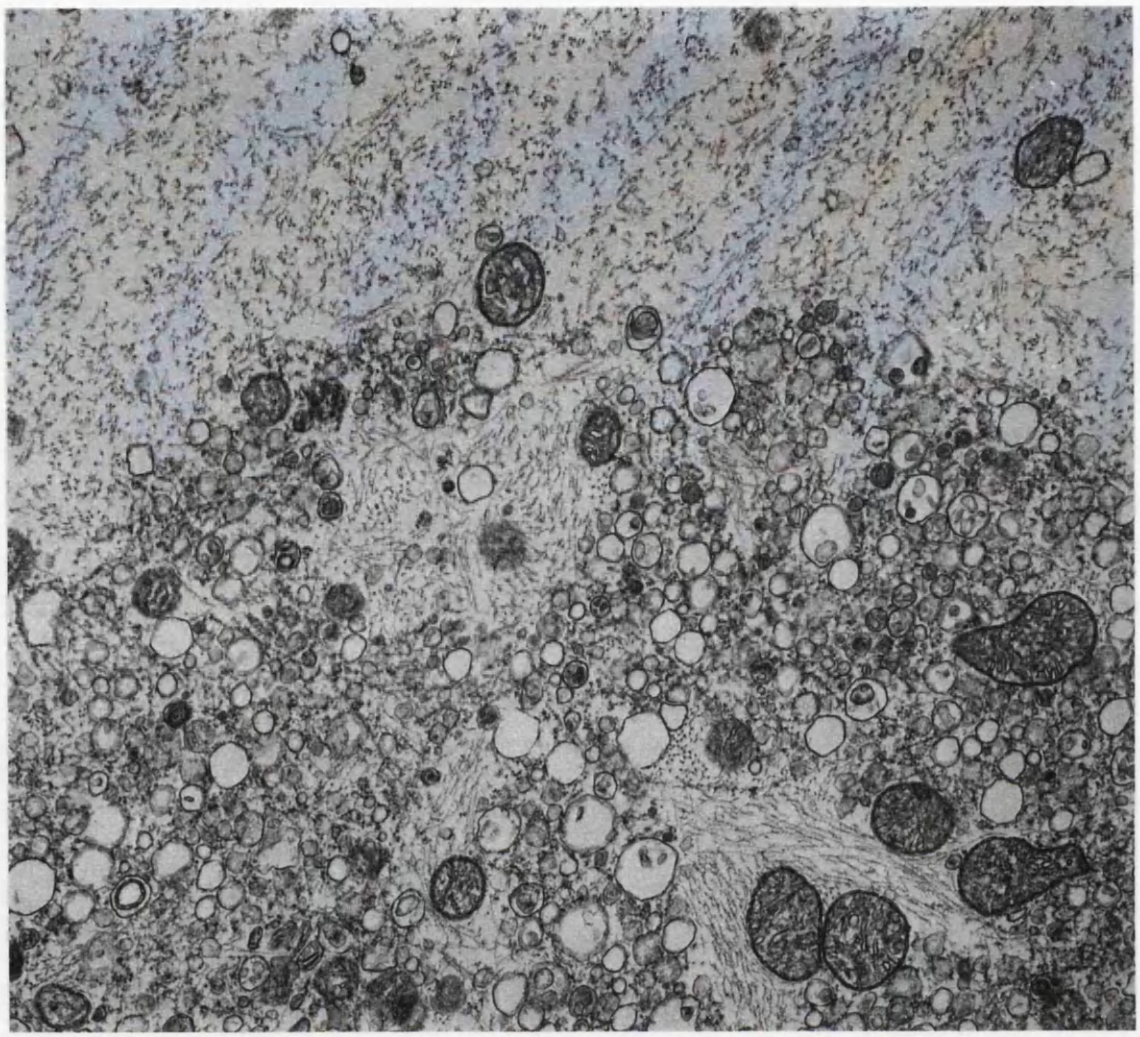


Figure 15c. Electronmicrograph of a neurone from the red nucleus of a dog with CDRM (c) the central portion of the dendrite shown in Figure 15 (a) to illustrate the accumulation of organelles. There are mitochondria, vesicles and vacuoles. Magnification approximately x20000.

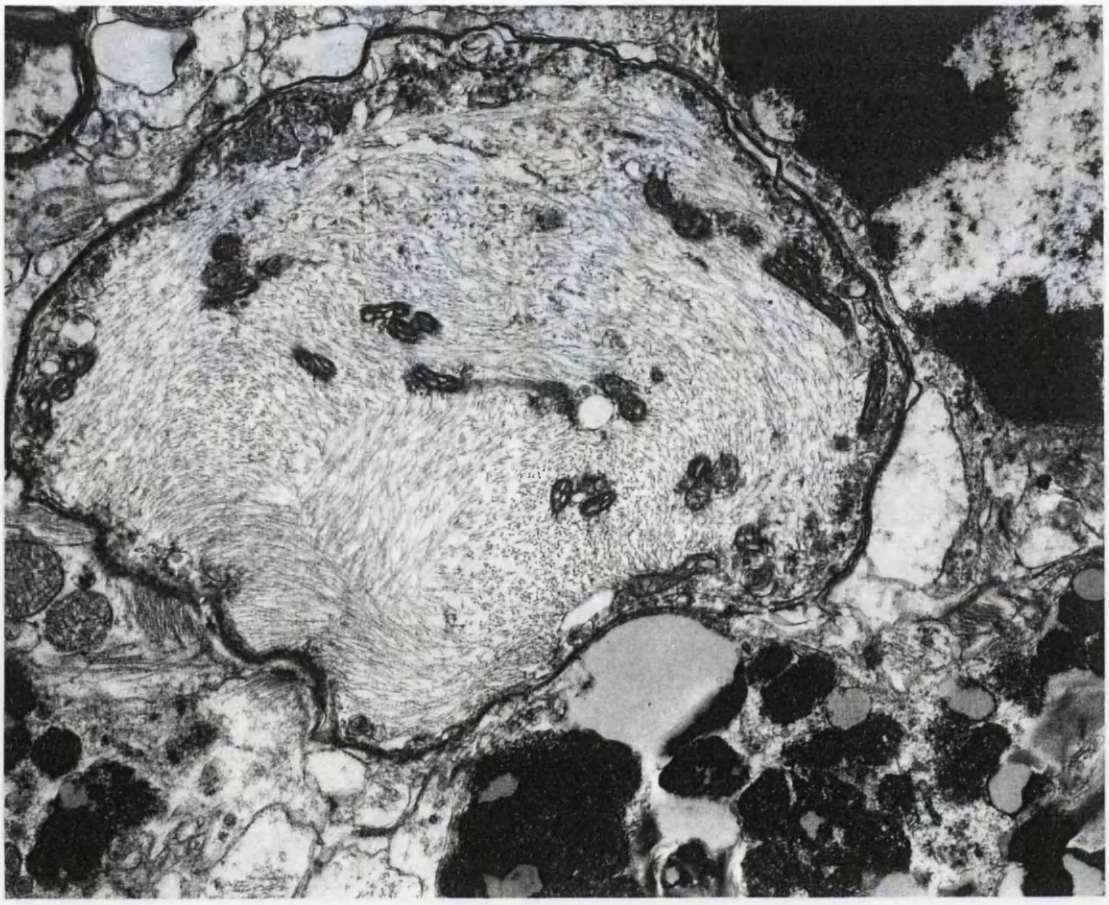


Figure 16a. Electronmicrograph of an axon from the red nucleus of a dog with CDRM (a) The axon, which is surrounded by an attenuated myelin sheath, contains disorganised neurofilaments. Magnification approximately x14000.

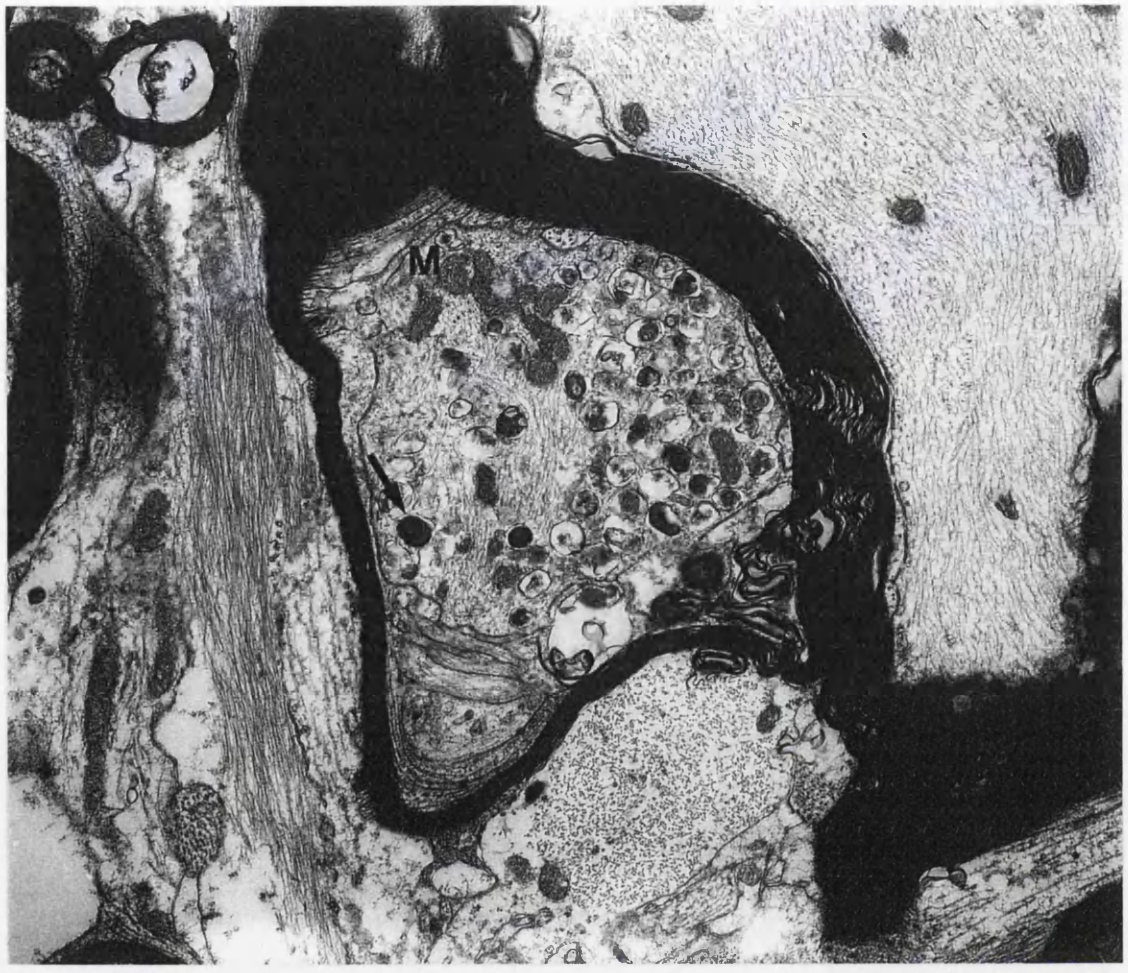


Figure 16b. Electronmicrograph of an axon from the red nucleus of a dog with CDRM (b) shows an axon containing many membranous organelles, mainly dense bodies (arrow) and mitochondria (M). Magnification approximately x14500.

5.3.13 Immunocytochemistry of the spinal cord of dogs with CDRM

Six of the spinal cords from severely affected dogs (Dogs 144, 5, 164, 163, 12, 49) were examined using a number of antibodies detailed in Table 3 (page 54, PAP technique), Table 4 (page 55, ABC technique) and Table 5 (page 56, double immunofluorescence).

5.3.13.1.1 White matter

As described above, the spinal cord white matter contained degenerating myelinated fibres with a concurrent gliosis. In general, the immunostaining profile supported this finding. Staining for axons using SMI 31 (Figure 17, page 147), RM024 or RT-97, or for myelin using α-PLP, showed varying severities of loss of myelinated axons. GFAP immunostaining was somewhat increased in the more severely affected regions (Figure 18, page 148).

5.3.13.1.2 Grey matter

Occasional swollen axons were seen using the SMI 31 and RM024 antibodies. Very rare examples of immunostained neuronal perikarya were present in all dogs.

5.3.14 Immunocytochemistry of the spinal cords of dogs with focal spinal cord lesions

A group of five dogs with focal spinal cord lesions were selected for immunocytochemistry (175, 172, 169, 177 and 179). These dogs had all shown some evidence, on routine histology, of secondary ascending and descending degeneration. The reduced immunostaining with SMI 31 and RM024 antibodies reflected the degree of axonal loss.

One case (Dog 171) had a brachial plexus avulsion of 7 days duration with typical chromatolytic changes in the ventral horn neurones of the cervical enlargement. The majority of neuronal somata in this region showed very prominent immunostaining with SMI 31 and RM024 antibodies.

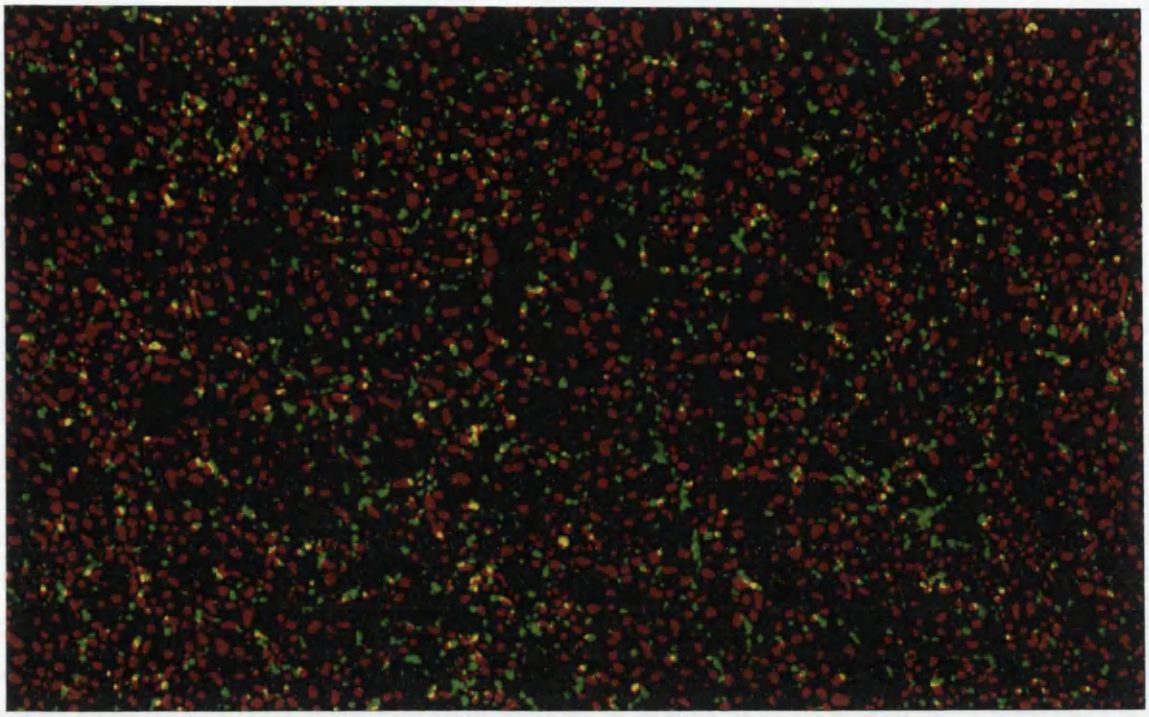
5.3.15 Immunocytochemistry of the spinal cords of neurologically normal dogs

Very occasional SMI 31-positive swollen axons were detected in the white matter and/or grey matter of spinal cords from neurologically normal dogs.

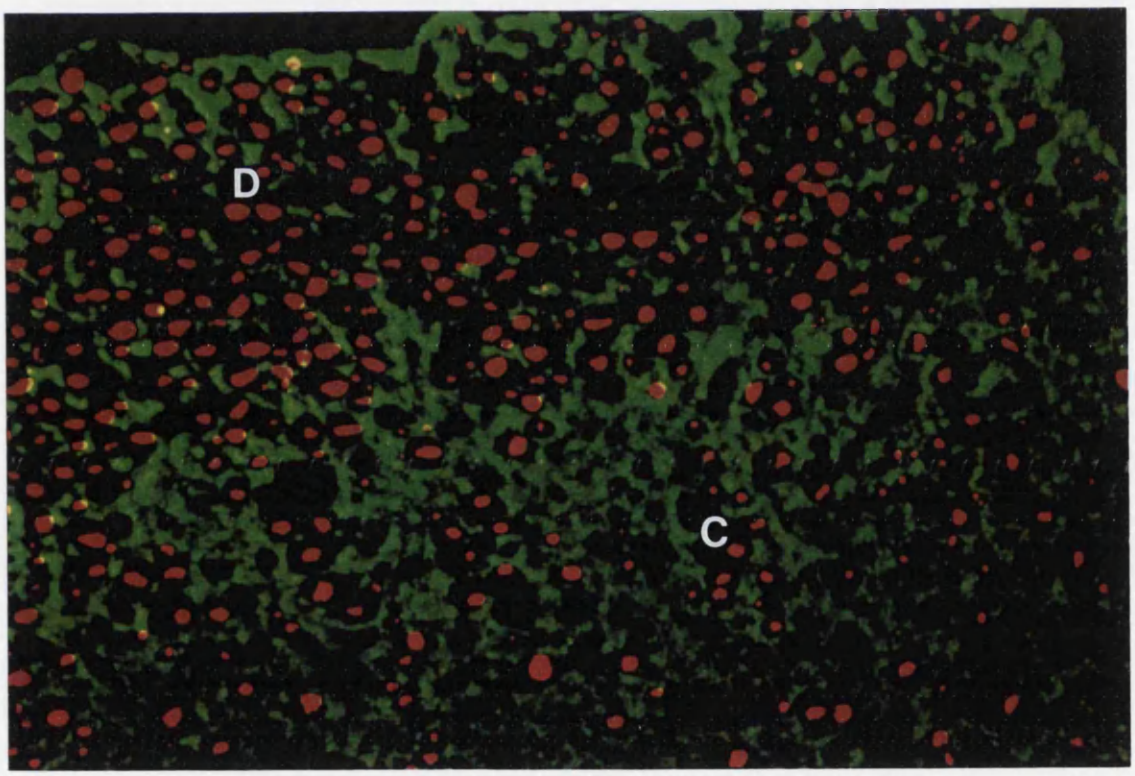
(a)

(b)

Figure 17. Section of lumbar spinal cord from a CDRM case immunostained with SMI 31 to show asymmetrical axon loss in the dorsal columns (arrow) (a) Magnification x43 and (b) Magnification x105. (a) and (b) L2



(a)



(b)

Figure 18. Confocal images of spinal cord immunostained for neurofilaments (red) and GFAP (green) (a) normal dog (b) a CDRM case to show the dorsal spinocerebellar tract (D) and underlying corticospinal tracts (C). The spinocerebellar tract has normal relationship between axons and astrocytes while there is axonal loss and astrocytosis in the corticospinal tract.
(a) and (b) T12 (a) x250 (b) x600

5.3.16 Immunocytochemistry and histochemistry of the red nucleus of dogs with CDRM

As the red nucleus was the most consistently and severely affected of the brain nuclei on histologic examination, it was examined using a number of the antibodies listed in Table 3 (page 54).

SMI 31 (Figure 19c, page 150) and RM024 antibodies revealed axonal swellings within the red nucleus of three cases (144, 49 and 12). Again using SMI 31, RM024, and RT-97, dog 5 had positively stained neurones which suggested an increase in phosphorylated neurofilaments throughout the neuronal perikarya. Dog 49 had positive staining with these antibodies which involved only the neuronal nuclei, which suggested a more localised increase in phosphorylated neurofilaments. In no case was there evidence of a dramatic astrogliosis or astrocytosis when GFAP was used.

Ubiquitin (Dako) immunocytochemistry was applied to the red nucleus of five affected cases (Dogs 2, 163, 19, 4 and 14) to determine whether the red nucleus of dogs with CDRM had evidence of filamentous intraneuronal inclusions, since these had been found in a number of chronic neurodegenerative diseases in man (Perry *et al.*, 1987; Mayer *et al.*, 1991). Two cases had positive staining of occasional neuronal nuclei (Dogs 2 and 4) while one (Dog 19) had staining of the nucleus only in some neurones and entire neuronal perikarya in others. The final two cases had generalised staining of several neuronal perikarya (Dogs 163 and 12). All cases examined had a random arrangement of small brown positively stained structures throughout the red nucleus. However, these changes were also found in the oculomotor nucleus.

A preliminary study of staining of red nucleus sections with Congo Red suggested that there was no amyloid deposition, which has been found in patients with Huntington's disease (Scherzinger *et al.*, 1997).

5.3.17 Immunocytochemistry of the red nucleus of neurologically normal dogs

No abnormalities were found.

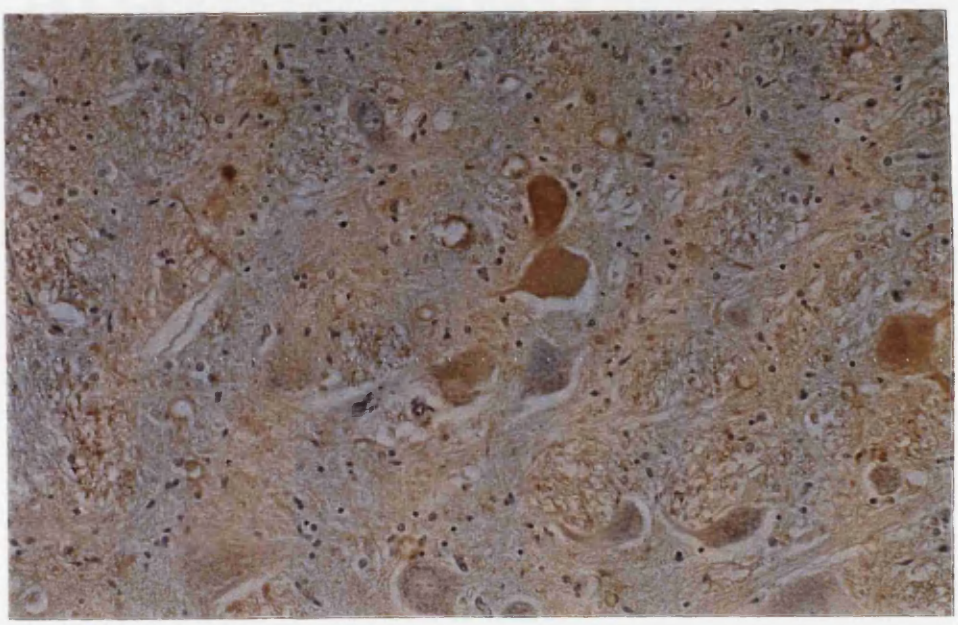
Figure 19. Sections immunostained with SMI 31. (a) red nucleus of a dog with a C6/7 lesion of three weeks duration showing that the majority of neuronal cell bodies contain phosphorylated neurofilaments. Occasional neuronal cell bodies show eccentric nuclei. (b) oculomotor nucleus from the same dog showing a neurone population with no immunostaining of the cell bodies. x120. (c) red nucleus from a dog with CDRM showing occasional immunopositive neurones. x120.

Facing page 150

(a)

(b)

(c)



5.3.18 Immunocytochemistry of the red nucleus of dogs with focal spinal cord lesions

Six cases were stained with SMI 31, RM024 and RT-97; one case showed no abnormalities (Dog 169), another had immunostaining in several neuronal nuclei (Dog 179). The most common finding was staining of the neuronal perikarya of one neurone (Dog 172) or several neurones (Dogs 174, 175 and 177) within the red nucleus; this was especially marked in dogs 175 (Figure 19a and b, page 150) and 177 where all the ventrolateral neurones in both red nuclei were positively stained with SMI 31. In addition, dog 177 also had evidence of axonal swellings when stained with SMI 31. Staining with GFAP showed no obvious astrocytosis in any of these dogs.

The case with the brachial plexus avulsion showed occasional staining of phosphorylated neurofilaments in neuronal nuclei with SMI 31. However similar changes were also seen in the oculomotor nucleus.

Ubiquitin staining was carried out on four dogs in this group (Dogs 169, 176, 170 and 174). One case (Dog 170) had no visible staining. The remaining three had some neurones with staining in the nucleus only, other neurones with staining throughout the perikarya while many neurones remained unstained. As with the CDRM cases, small brown structures were seen throughout the section and changes seen in the red nucleus were also seen in the oculomotor nucleus.

Histological staining with Congo Red showed the occasional orange stained area in the neuronal perikarya (Dog 169) which suggested possible amyloid deposition.

5.4 Discussion

In the present study spinal cord white matter changes which comprised degenerating axons with an associated loss of myelin, often with gliosis, were found in the dorsal, lateral and ventral funiculi of affected dogs. The numbers of degenerate fibres in specific tracts varied both throughout the length of each cord and between cases. Both ascending and descending tracts were affected, which correlated with the clinical finding of both sensory and motor deficits. The most consistently affected regions of the white matter were the corticospinal tract and rubrospinal tract in the lateral funiculus. As previously discussed (see 1.3.3.6 Rubrospinal tract, page 17), the rubrospinal tract is an important motor tract while the lateral corticospinal tract is of much less functional significance in dogs than in man. These two tracts were difficult to distinguish as the fibres intermingle to some extent, and are often considered together as the corticorubrospinal tract. Degeneration in the rubrospinal

tract would result in difficulties with coordination and possibly locomotion. Regions with the greatest area of involvement were the caudal thoracic and lumbar segments which agreed with previous reports (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978). This study did not confirm the occurrence of small vascular elements of increased density which had been reported by Clemmons (1989).

The distribution of the degeneration in the dorsal funiculi was less consistent than that in the lateral funiculi. The majority of cases showed degenerating fibres in the deeper regions of the dorsal funiculus of the caudal lumbar segments. At the level of the mid to caudal thoracic segments very little change was observed in the dorsal funiculi. One possible explanation is that the degenerating fibres in lumbar and caudal thoracic segments represent the central projections of primary sensory neurones whose cell bodies lie in the lumbar SpG. The majority of such fibres terminate in the base of the dorsal horn of the grey matter within a few segments of entering the spinal cord (King, 1993). Thus it is the distal portions of such axons which degenerate. The other region of dorsal funiculus involvement was the fasciculus gracilis of the cervical cord, although this was an inconsistent finding. These fibres probably represent the long projections of neurones whose cell bodies also lie in the lumbar SpG. Thus both regions of degeneration in the dorsal funiculi may represent the distal portions of axons whose cell bodies lie in the lumbar SpG.

Gliosis in the grey matter of the spinal cord of cases of CDRM has been reported previously (Averill, 1973; Braund and Vandevelde, 1978). In the present study, the distribution of gliosis varied between cases but most commonly affected the intermediate horn of the grey matter. It was striking that only the middle and caudal lumbar segments were consistently affected by the gliosis in the intermediate grey matter. One possible explanation for this distribution of gliosis is that it is related to the terminations of the fasciculus gracilis and the descending tracts which were affected by the degeneration. Both the corticospinal and vestibulospinal tracts have been reported to terminate on interneurones found within this region (Jenkins, 1978). There was no gliosis in the substantia gelatinosa which agreed with the clinical finding that pain and temperature detection were unaffected in cases of CDRM. Occasional chromatolytic neurones were found in the grey matter of affected dogs, most commonly in the intermediate horn.

The finding of occasional chromatolytic neurones in the SpG of some affected cases agreed with a previous report (Griffiths and Duncan, 1975a). Occasional chromatolytic neurones were also seen in a number of the dogs with focal spinal cord lesions. Of the normal dog SpG examined, only one had a single chromatolytic

neurone. These findings suggest that such degeneration in the SpG was not exclusively a change seen with CDRM but was indicative of spinal cord pathology. Vacuolation seen in the nerve roots of some of the affected dogs and a number of the control dogs could be an age-related change as discussed by Griffiths and Duncan (1975b).

Clemmons (1989) is the only author who has previously mentioned changes in the brain of affected dogs. He reported occasional lesions in unspecified areas of the white matter of the brain, consisting of demyelination and axon loss, with an associated increase in astrocyte numbers and increase in density of small vascular elements. In the present study, serial sectioning of the brain of affected dogs revealed changes in a number of specific brain nuclei and white matter tracts. Brain nuclei affected were the red nucleus, LVN and lateral nucleus. The severity of changes varied with each affected dog but in all cases the red nucleus was most consistently and severely affected. Evidence of degeneration included neurones with eccentric nuclei and chromatolytic neurones, sometimes with an associated gliosis. The red nucleus also had a variable loss of neurones in the more severely affected dogs, suggesting that the chromatolytic neurones in milder cases were degenerating and would subsequently be lost. Evidence of degeneration was also found in the ventral tegmental decussation, consisting of gliosis and occasional degenerating fibres. Involvement of the red nucleus was consistent with the clinical signs since it functions as an important motor centre and as a relay station for proprioceptive impulses from the cerebellum to the cerebral cortex.

Although an extensive immunostaining profile was performed, it added relatively little to the knowledge gained from paraffin sections stained with H&E or Cresyl violet. Any changes found, such as presence of phosphorylated neurofilaments in neuronal cell bodies of the brain nuclei, or ubiquitin staining of these cells, tended to be inconsistent and involved only a small number of neurones The ultrastructural examination supported the light microscopic analysis. The loss of Nissl granules and dispersion of the RER would account for the chromatolytic appearance of neurones. The ultrastructural studies also showed that a small proportion of axons in the red nucleus were abnormal with accumulation of neurofilaments and organelles. Although the association of such axons with neurones of the red nucleus could not be confirmed, it seemed most probable that this was their origin.

Changes in the red nucleus could result from one of two basic mechanisms (i) the primary abnormality affected the neuronal cell body with consequent degeneration of the axonal projections to the spinal cord or (ii) the primary changes occurred within the spinal cord and the findings in the red nucleus represented a retrograde

axonal response. It was also possible that a combination of these events might have occurred such that a derangement of metabolism in the neuronal cell body compromised the transport of material into the spinal axon which consequently degenerated, leading in turn to a retrograde axonal response in the parent cell body. In order to try and distinguish these possibilities the red nucleus was studied from dogs with focal spinal cord lesions of known duration. In this group of dogs the red nucleus was much less consistently and much less severely affected. Two of the cases with spinal cord lesions *per se* had changes, one had occasional chromatolytic neurones with no associated gliosis while the other had one neurone with an eccentric nucleus. The only other dog in this group with changes in the red nucleus had a brachial plexus avulsion. There were two chromatolytic neurones in the red nucleus of this dog. None of these dogs had any obvious evidence of neuronal loss. In the group of dogs with focal spinal cord lesions there was very little degeneration in the red nucleus. Additionally there was no evidence of degeneration in the ventral tegmental decussation. These findings suggested that a primary spinal cord lesion alone would not result in the degree of degeneration seen in the red nucleus of dogs with CDRM.

Ideally, the control tissue would have been obtained from age-matched dogs of large breeds which had had chronic thoracolumbar spinal cord lesions of a similar time course to CDRM. Due to the constraints imposed by using clinical cases, such material was not available. Thus the material collected was from those dogs with focal spinal cord lesions of longest duration that were euthanased at GUVS during the course of this project. The most longstanding lesion had resulted in clinical signs of three weeks duration. This discrepancy of duration was taken into consideration when comparing the two groups.

Since extensive neuropathology has been carried out on the spinal cords of humans with chronic spinal cord lesions, a literature search was completed for further comparisons with the CDRM cases. Following traumatic damage to the spinal cord in man (Kakulas, 1987; Fishman, 1987) retrograde axonal damage was limited to a certain distance such that patients with low or mid-thoracic lesions had apparently normal corticospinal tracts by the mid-cervical level, while patients with a high thoracic to low cervical lesion had apparently normal corticospinal tracts by the upper cervical level. The medullary pyramids were unaffected in all cases examined, which suggested that retrograde damage, even from a severe traumatic lesion, did not extend into the brain itself. The extent of axonal damage in the corticospinal tract cranial to the lesion in patients with spinal cord injury decreased with increasing distance from the lesion. If retrograde damage followed a similar pattern in the dog it was unlikely that such damage to axons in the caudal thoracic

segments would extend as far cranially as the ventral tegmental decussation. Since evidence of degeneration was found in this structure in a number of the CDRM cases it was possible that the primary lesion was in fact the neuronal damage in the red nucleus. However, on the basis of the pathology it was not possible to unequivocally identify the primary site of the defect.

Selective damage to the thoracic spinal cord had been reported in vitamin B_{12} deficiency in humans and dogs. Vitamin B_{12} deficiency in humans (Metz, 1992) resulted in a degenerative lesion characterised by swelling of myelin sheaths and breakdown of myelin with disruption of the axon, leading to a spongy, vacuolated cord. Changes were most severe in the dorsal funiculi of the thoracic cord, although the lateral funiculi did become affected later (subacute combined degeneration). In addition to the spinal cord lesions, foci of demyelination were also found in unspecified areas of the white matter in the brain. This condition also affected the peripheral nerves which showed a decreased number of myelin sheaths. An inherited B_{12} deficiency had been reported in the Giant Schnauzer dog (Fyfe *et al.*, 1991) which had a simple autosomal recessive inheritance, and closely resembled the human condition in clinical presentation and pathology. The underlying mechanism for this disease was not properly understood but was thought to be a failure of ileal enterocytes to mediate transcytosis of cobalamin. There were significant differences between the pathology associated with vitamin B_{12} deficiency and that seen in CDRM. The pathology seen in the thoracic cord was originally perivenular in the vitamin B_{12} deficiencies and was usually multifocal whereas the lesions in CDRM were topographically continuous in all tracts in most cases (an exception being the dorsal funiculi in a small number of affected dogs). In addition, the peripheral nervous system (PNS) remained unaffected in CDRM. The available evidence would suggest that CDRM is not the result of a deficiency of vitamin B_{12}.

Vitamin E has been tentatively linked with the aetiopathogenesis of CDRM. Deficiency of this vitamin has been associated with neurological disease in a number of naturally occurring and experimentally induced conditions in several species. Following two years on a vitamin E deficient diet a group of rhesus monkeys (Nelson *et al.*, 1981) had a concomitant loss of axons and myelin sheaths in the dorsal columns accompanied by mild fibrillary astrocytosis. Prolonged deficiency in rats (Pentschew and Schwarz, 1962; Southam *et al.*, 1991) resulted in systemic axonal dystrophy in rostral parts of the dorsal columns and in the gracile and cuneate nuclei with secondary loss of myelin and accompanying Wallerian-type

fibre degeneration (changes which were typical of a central-peripheral distal axonopathy).

Vitamin E deficiency in man is very uncommon but does occasionally result from chronic fat malabsorption. This occurs in atresia of the bile ducts and other severe forms of chronic cholestasis in children or secondary to genetic disorders, for example, abetalipoproteinaemia which is the most severe naturally occurring form of vitamin E deficiency. The resulting multisystemic disorder has a neurological component. One distinct clinical and genetic entity which has led specifically to spinocerebellar degeneration was called ataxia with vitamin E deficiency (AVED). In this condition, the pathology was the result of impaired hepatic secretion of α-tocopherol into blood, which resulted in plasma vitamin E levels which were approximately 10% of normal. The resulting neurological deficit was a peripheral neuropathy caused by the dying back of large diameter fibres. The precise neuropathology associated with this condition had not been characterised but there did appear to be greater involvement of the central sensory pathways than the peripheral nerves (Ouahchi *et al.*, 1995; Arita *et al.*, 1995).

The neuropathology associated with vitamin E deficiency has been reported in much more detail in a number of conditions in the horse. Equine degenerative myeloencephalopathy (EDM) (for details see 1.5.2 Equine, page 24) resulted in widespread neuroaxonal dystrophy in brain stem nuclei, lateral cervical and thoracic nuclei and lumbosacral and cervical intermediate grey columns. In addition, lipofuscin pigment accumulation and astrogliosis were seen in these areas while the ventral and dorsal spinocerebellar tracts and ventromedial funiculi of the cervical and thoracic spinal cord segments showed degeneration of axons and myelin with some gliosis (Mayhew *et al.*, 1987). A form of neuroaxonal dystrophy had also been described in the Morgan horse which had pathologic changes only in the accessory cuneate nucleus. A spinal ataxia had been described in the zebra (Montali *et al.*, 1974) which was characterised by symmetrical degeneration in the ventral (descending tracts adjacent to the ventro-median fissure) and lateral (ascending and descending tracts) funiculi. Both the axons and myelin sheaths were affected and the degenerate tracts were topographically continuous. In this condition the dorsal columns and grey matter were unaffected and no changes were found in the brain rostral to the medulla oblongata. These conditions had some similarities with the pathology of CDRM, in that both the axons and myelin sheaths were degenerate in affected tracts and many of the same tracts were affected. However, the most severely affected white matter tracts in EDM were the dorsal and ventral spinocerebellar tracts while the corticospinal and rubrospinal tracts were the most severely affected in CDRM. Axonal swellings were a prominent feature in these

equine conditions while they occurred infrequently in CDRM. Although there were some similarities in pathology between the conditions caused by vitamin E deficiency and CDRM, on balance it was unlikely that vitamin E deficiency was a significant factor in the development of CDRM in the GSD.

Previous studies (Waxman *et al.*, 1980a; Waxman *et al.*, 1980b; Barclay and Haines, 1994) implicated a cell-mediated autoimmunity as a possible cause of CDRM (page 5 Introduction). In this study there was no evidence of perivascular cuffing nor of lymphocyte infiltration in the spinal cord in either paraffin or resin sections. As there was no other evidence from the pathology to suggest an immune-mediated mechanism this aspect was not pursued any further. An obvious way to definitively rule out the presence of inflammatory cells would be to use antibodies specific to each cell type *e.g.* T lymphocytes, B lymphocytes and macrophages.

Recent reports on the pathology of a group of human neurodegenerative diseases which have marked clinical and pathological similarities with CDRM suggested that ubiquitin positive fibrillar inclusions (Davies *et al.*, 1997; Scherzinger *et al.*, 1997; DiFiglia *et al.*, 1997) were found in the nuclei of specific neurones in both affected patients and in mice transgenic for the Huntington's disease mutation. Equivalent inclusions were not found in any of the CDRM cases examined. Amyloid had also been found (Scherzinger *et al.*, 1997) in the cytoplasm of affected neurones in Huntington's disease; to date this has not been found in the CDRM cases. The above changes had been reported in the areas of brain known to be the primary lesion. At this stage the red nucleus might well be the primary site of damage in CDRM, however this has not been established definitively which could explain the lack of such inclusions in the CDRM cases.

Examination of Figure 7 indicated that there was some correlation between the gait score at time of death with the extent of degeneration found. However, dogs with the same gait score did not consistently have the same pattern of degeneration on examination of the spinal cord. The pattern of pathological change was not consistent with the clinical signs.

In summary, CDRM is a neurodegenerative disorder which affects selected areas of the spinal cord and brain stem. The changes in the white matter were those of Wallerian-type degeneration which have a tendency to affect the more distal regions of certain motor and sensory tracts. In this sense CDRM had some features of a distal axonopathy ("dying back" disease). However, the abnormalities in brain nuclei such as the red nucleus, may indicate a primary problem in the neuronal somata as a result of which degeneration may occur in the distal regions of the

associated axons. Defining the primary site of the pathology *i.e.* neuronal cell body versus distal axon would require examination at the very earliest time point of the disease. The pathology identifies lesions in the red nucleus which could be consistent with change in the descending tracts. However there is no consistent obvious pathological change in the neuronal somata supplying the ascending tracts. This dichotomy may represent a useful area for further study. With the constraints imposed through the use of clinical cases such material would be very difficult to obtain. It is also probable that such changes could be subtle and resolvable only in material preserved by intravascular perfusion, again imposing considerable difficulties in collection.

6. An investigation towards the identification of a possible molecular lesion associated with CDRM

6.1 Background

The identification of CDRM as a neurodegenerative disorder prompted a search for similar diseases in other species, particularly man, in which an aetiology was known. Several unrelated human neurodegenerative disorders have late-onset and were associated with degeneration of specific neuronal populations, as occurred in CDRM. Many of these disorders were caused by expanded trinucleotide repeats, particularly the CAG repeat.

This section briefly reviewed aspects relevant to trinucleotide repeats and the neurodegenerative disorders which have been associated with expanded repeats.

6.2 Trinucleotide repeats in the mammalian genome

Classical studies based on reassociation kinetics have shown that the haploid mammalian genome contained a number of repeating nucleotide sequences. The size of these repeats varied from complex complete genes (such as the ribosomal RNA genes) down to simple sequences of one or two base pairs. Organisation of these repeat sequences varied from widely dispersed copies of a relatively long, complex sequence to tandem arrays of simple sequence composition. Arrays of up to five to six nucleotides were called simple tandem repeats (STRs). Within this group dinucleotide repeats were the most common, most notably AC.

Of particular interest were the expanded trinucleotide repeats, which could occur at various loci throughout the transcription unit. Increase in the length of these repeats had arisen in a number of human disorders. The first genetic disease reported in association with a trinucleotide repeat was Fragile X Syndrome which was the result of the dynamic mutation of the sequence CCG in the 5/ untranslated region (UTR) of the *FMR1* gene and was the most common form of familial mental retardation (Sutherland and Richards, 1995). Since then a number of other diseases have been linked to expanded repeats and are presented in Table 31 (page 161).

Disease	Expanded Repeat
various human cancers	minisatellite, di-,tri- & tetra-nucleotides
Hereditary Non-polyposis Colon Cancer	mono-,di- and tri-nucleotide
Fragile X	CCG
FRAXE	CCG
Spinal and bulbar muscular atrophy	CAG
Myotonic dystrophy	CTG
Huntington`s disease	CAG
Spinocerebellar ataxia 1-7	CAG
Machado-Joseph disease	CAG
Haw river syndrome	CAG
Dentatorubral-pallidoluysian atrophy	CAG
Friedreich`s ataxia	GAA

Table 31. Human diseases which have been associated with expanded repeats.

6.2.1.1 Trinucleotide repeats

Trinucleotide repeats have been found in all regions of the transcription unit *viz.* the 5/ non-coding region, exons, introns or the 3/ non-coding region. Diseases caused by trinucleotide repeat expansions fell into two major groups. The first group, which have been classified as Type I disorders (Stuart and Leipold, 1983; La Spada *et al.*, 1994; Perutz, 1996; Sanjeeva Reddy and Housman, 1997), included the progressive neurodegenerative disorders; X-linked spinal and bulbar muscular atrophy (SBMA), Huntington`s disease, Dentatorubral-pallidoluysian atrophy (DRPLA), Machado-Joseph disease (MJD) and the Spinocerebellar ataxias (SCA1-7). These disorders were characterised by neuronal loss in specific brain nuclei and essentially affected only the nervous system, with the exception of SBMA, which also had systemic hormonal effects. They were caused by small, limited CAG repeat expansions, normal individuals had up to 37 repeats while those with disease always had more than 41 repeats, which encoded polyglutamine (Figure 20, page 163). These mutations altered properties of the affected protein which resulted in a

gain of function which was particularly deleterious to neurones. The second group called Type II disorders included Fragile X syndrome (FRAXA), FRAXE mental retardation and Myotonic dystrophy (DM). These were all multi-system disorders which were associated with much larger expansions of repeats located in the 5/ (FRAXA, FRAXE) or 3/ UTR (DM) of the gene. Diseases in the second group were not progressive neurodegenerative disorders but may caused abnormal brain and neuromuscular development. In addition, this group exhibited marked somatic mosaicism, with repeat sizes which varied widely throughout the somatic tissues of affected individuals.

For both these groups, when an expansion resulted in disease, the disorder transmitted as a dominant trait subject to genetic anticipation. This feature was thought to be due to the inherent instability of the expanded trinucleotide repeat, which could alter in size in successive generations (Sutherland and Richards, 1995). The length of repeats could contract, but much more commonly would increase in unit length as it passes through the germline, which resulted in an earlier age of onset and a more severe phenotype in successive generations. In many of these diseases, although not in DM, the paternal line was more unstable than the maternal, presumably due to the larger number of cell divisions which occurred per generation (Jennings, 1995; Paulson and Fischbeck, 1996). Recent studies of individual sperm from males with Huntington's disease had demonstrated a significant increase in sperm trinucleotide repeat lengths compared with the repeat lengths of the father (Sanjeeva Reddy and Housman, 1997). In addition, repeat length increased as a function of paternal age. These findings suggested that replication of DNA in primary male germ cells may either favour repeat expansion or select for germ cells or mature sperm which carry longer repeat expansions. Expansions arose from CAG triplet repeats, which were polymorphic in the normal population (Warren, 1996; Paulson and Fischbeck, 1996), but once a critical threshold was reached, which varied according to the condition, hairpin structures could form which would result in replication errors and increased repeat size. The number of repeats correlated more closely with age of onset than severity of clinical signs (Richards and Sutherland, 1994).

Another neurodegenerative disease was Friedreich's ataxia (Stumpf *et al.*, 1987; Ben Hamida *et al.*, 1993; Campuzano *et al.*, 1996; Carvajal *et al.*, 1996). This condition was most often manifest from adolescence although occasionally onset in middle age or later occurs. The molecular lesion was a GAA repeat expansion within intron 1 of the *X25* gene which coded for the protein frataxin. Unusually the mode of inheritance was autosomal recessive, meaning that anticipation could not be seen as one generation only was affected (Mandel, 1997).

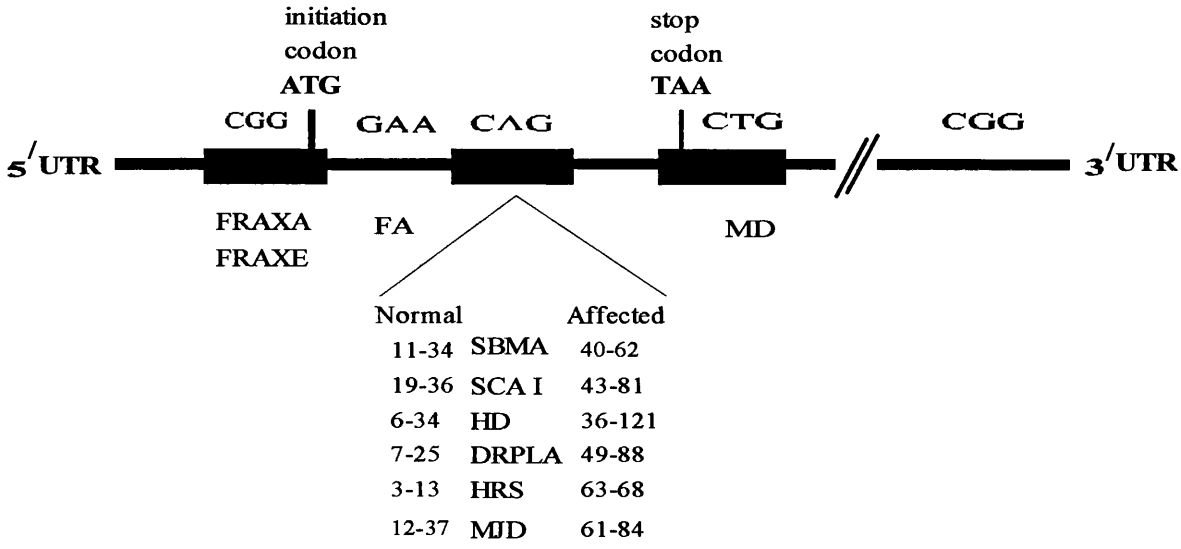


Figure 20. Schematic representation of an archetypal Pol II gene depicting the loci of the lesions for a number of the human conditions associated with expanded trinucleotide repeats.

6.2.2 Neurodegenerative conditions resulting from CAG repeat expansions

The spinocerebellar ataxias and DRPLA had several similarities to CDRM in terms of pathology and were probably the most relevant conditions. Huntington's disease and SBMA were described for completeness but appear to had few pathological similarities with CDRM.

6.2.2.1 Huntington's disease

Huntington's disease, the most commonly occurring Type I disease (Paulson and Fischbeck, 1996), was an autosomal dominant neurodegenerative disorder characterised by involuntary movements (chorea), behavioural disturbance and cognitive impairment. Early in the disease there was selective degeneration of striatal projection neurones which progressed to widespread neuronal loss in advanced cases. The genetic defect, an expanded $(CAG)_n$ repeat within the coding region of the large *IT-15* gene, was identified in 1993 by the Huntington's Disease Collaborative Group. The product was huntingtin, the CAG expansion encoded a polyglutamine tract six to 34 amino acids long in normal individuals and 36 to 121 in Huntington's disease patients (MacDonald and Gusella, 1996).

6.2.2.2 X-linked spinal and bulbar muscular atrophy (SBMA)

X-linked spinal and bulbar muscular atrophy or Kennedy's Disease was a rare progressive adult-onset motor neuronopathy which was characterised by proximal muscle weakness, atrophy and fasciculations. Pathological changes seen were degeneration of the anterior horn cells, sensory neurones in the SpG and brain stem motor nuclei (Brooks and Fischbeck, 1995). The trinucleotide repeat $(CAG)_n$ in the first exon of the androgen receptor (*AR*) gene was expanded from the normal range of 11 to 34 residues to 40 to 62 in SBMA patients.

6.2.2.3 Spinocerebellar ataxias 1-7

These late-onset progressive neurodegenerative diseases were dominant spinocerebellar ataxias, which represented a phenotypically heterogeneous group of disorders, with a prevalence of familial cases of approximately 1 in 100,000. They were all characterised by varying abnormalities of balance attributed to dysfunction or pathology of the cerebellum and cerebellar pathways. In many of these conditions dysfunction extended beyond the cerebellum to involve basal ganglia function, oculomotor disorders and neuropathy.

6.2.2.4 Spinocerebellar ataxia 1 (SCA1)

Spinocerebellar ataxia 1 was characterised by ataxia, ophthalmoparesis and weakness, sometimes with spasticity, which typically began in the third to fifth decade and progressed over 10 to 20 years. Anticipation could result in a much earlier onset and more rapid progression, childhood cases most often resulted from paternal transmission. The disease usually began with cerebellar signs such as clumsiness and ataxia but progressed to include dysarthria, bulbar dysfunction, oculomotor disturbance and pyramidal tract signs. Neuropathological changes included loss of Purkinje cells and the neurones in the inferior olive, with degeneration of the spinocerebellar tracts. The molecular lesion was in the protein-coding region of the *SCA1* gene which encoded the protein ataxin-1. The variation in ataxin-1 polyglutamine tract length was from 19 to 36 repeats in normal individuals to 43 to 81 repeats in affected individuals. The expanded repeat was invariably an uninterrupted sequence of CAG repeats. In contrast to the normal-sized CAG alleles which were almost always interrupted by one to three CAT repeats (La Spada *et al.*, 1994). It was suggested that this difference may contribute to the stability of the normal allele.

6.2.2.5 Spinocerebellar ataxia 2 (SCA2)

Patients with SCA2 were often clinically indistinguishable from those with SCA1. Additional clinical signs seen in some SCA2 patients included dementia and neuropathy. The brain regions most affected by pathology were the cerebellum, pontine nuclei, inferior olives and substantia nigra (Imbert *et al.*, 1996). In this disorder the molecular lesion was an expanded CAG repeat in the spinocerebellar ataxia 2 (*SCA2*) gene on chromosome 12 which encoded ataxin-2. Normal individuals had 15 to 24 repeats while affected individuals had 35 to 59 repeats.

6.2.2.6 Spinocerebellar ataxia 3 (SCA3)

Despite having a phenotype which was distinct from that of Machado-Joseph disease, the genotype in the two diseases was identical, both having an expanded CAG repeat in the gene variously called *SCA3* (Zoghbi, 1996) and *MJD1* (Haberhausen *et al.*, 1995). Clinically these patients showed severe cerebellar dysarthria and supranuclear ophthalmoplegia with ataxia of limbs and gait. Neurodegeneration involved the cerebellar peduncles, dorsal columns and basal ganglia predominantly although some affected families also demonstrated loss of Purkinje cells. Normal individuals had 13 to 36 repeats while SCA3 patients had 66 to 79 (Matilla *et al.*, 1995).

6.2.2.7 Spinocerebellar ataxias 4, 5 and 7 (SCA4, 5, and 7)

These conditions were clinically indistinguishable and characterised by ataxia, dysarthria, dysmetria, intention tremor and macular degeneration leading to blindness. On neuropathological investigation, degeneration of the pathways associated with the cerebellum was found. The exact position of the CAG expansion remained to be defined for these conditions although the chromosomal locus in SCA4 and SCA5 had already been identified: SCA4; 16q24 and SCA5; 11cen.

6.2.2.8 Spinocerebellar ataxia 6

SCA6 was characterised by ataxia, dysarthria, nystagmus and vibratory sense loss. Cerebellar and mild brain stem atrophy were seen. The affected gene encoded ataxin-6 (a calcium channel subunit, α_{1A}). This condition was unusual in that normal individuals had 4 to 16 repeats while affected patients had a relatively small expansion with only 21 to 27 repeats.

6.2.2.9 Machado-Joseph disease (MJD)

MJD could be separated into three distinct subtypes on the basis of age at onset and clinical presentation (DeStefano *et al.*, 1996). Type II was the most frequent presentation with progressive ataxia, pyramidal signs and ophthalmoplegia. The mean age of onset for this group was approximately 40 years of age. Type I was characterised by early onset and had marked pyramidal and extra-pyramidal signs in addition to the signs seen in type II. Type III was also associated with marked distal atrophy and sensory loss and tended to develop between 40 and 60 years of age. The molecular lesion was an expanded CAG trinucleotide repeat sequence in the Machado-Joseph disease 1 (*MJD1*) gene on chromosome 14 which encoded ataxin-3. Normal individuals had 12 to 37 repeats while affected patients had between 61 and 84 repeats (Paulson *et al.*, 1997).

6.2.2.10 Dentatorubral-Pallidoluysian atrophy (DRPLA)

DRPLA was a rare neurodegenerative disease characterised by ataxia, choreoathetosis, myoclonus, epilepsy and dementia. Clinically, this condition had been divided into three subtypes; myoclonic-epilepsy, pseudo-Huntington's and ataxic-choreoathetoid forms. However, the distinctions between these types were not precise and more than one form could be seen within the same family. DRPLA showed anticipation, the most severe cases usually resulted from paternal transmission. Patients which exhibited early onset usually progressed more rapidly and had myoclonic epilepsy. Neuronal degeneration was frequently widespread and

particularly severe in the globus pallidus, dentato-rubral system and subthalamic nucleus (Luys body). The CAG repeat in the *CTG-B37* gene encoded a polyglutamine tract in a protein predicted to be 124kDa. This gene was one of a family which were expressed specifically in the CNS. Paulson and Fischbeck (1996) reported that normal individuals had 7 to 25 copies of the repeat compared with 49 to 88 copies in affected patients.

6.2.2.11 Haw river syndrome (HRS)

A condition with marked clinical and pathological similarities with DRPLA, Haw river syndrome, was an autosomal dominant neurodegenerative disease characterised by ataxia, chorea, seizures and dementia. There was a marked neuronal loss in the dentate nucleus, microcalcification of the globus pallidus, demyelination of the centrum semiovale and marked neuroaxonal dystrophy of the posterior columns (Burke *et al.*, 1995). This specific condition had been seen in only one African-American family where it started between 15 and 30 years of age and progressed to death within 15 to 25 years. The molecular lesion involved a CAG expansion of the *CTG-B37* gene. Burke *et al.* (1995) found three to 13 repeats in normal individuals with patients having 63 to 68 repeats (these figures varied from those given for normal individuals quoted in the previous paragraph, however both ranges were well below the threshold for disease). Due to the geographical distance between the Japanese families with DRPLA and this HRS family it was likely that HRS had occurred as a spontaneous mutation.

Despite a number of neurodegenerative diseases being reported which shared extended polyglutamine stretches in the altered proteins, the pathogenesis of the neuropathology was still controversial. Based on the phenotypic similarities between these diseases there appeared to be a common mechanism at work. In addition, recent work had led to the identification of proteins which interacted with polyglutamine peptides, irrespective of the specific flanking amino acids (Pulst *et al.*, 1996). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was bound by a polyglutamine peptide, both huntingtin and atrophin-1 (the DRPLA gene product) bind to GAPDH *in vitro*. A binding protein had been identified which appeared to interact specifically with huntingtin and not with atrophin-1. Although huntingtin was widely expressed, this huntingtin-associated protein showed a pattern of expression restricted to cell types involved in Huntington's disease and may have explained the cell-type specific degeneration seen in the disease.

6.3 Perspective

A number of late onset neurodegenerative diseases in humans bore resemblances to CDRM. Several of these autosomal dominant diseases *viz.* SCA I, DRPLA, MJD and Huntingtons Disease correlated with the presence of a CAG trinucleotide repeat expansion which encoded a polyglutamine tract. To date, all diseases which corresponded to a trinucleotide repeat expansion had been restricted to humans. All were late-onset disorders characterised by degeneration of specific (though different in each case) neuronal populations. In addition, there was considerable heterogeneity even within a single disease. It was remarkable that so many late-onset neurodegenerations were caused by the same basic mutation *viz.* a CAG expansion. Based on a combination of the high incidence of CDRM in the GSD population and the clinical and pathological similarities between CDRM and the human disorders an investigation into whether CDRM in the dog was also associated with a CAG trinucleotide repeat expansion was begun. It must be emphasised that although CDRM affected specific neuronal populations, the distribution of the neurodegeneration in CDRM was not identical to any of the aforementioned human neurodegenerative disorders.

Such a search raised two main factors which merit close attention. One inherent difficulty was the late age of onset of CDRM as compared to the expected life span. Dogs can develop clinical signs at any time from six years up to 14 years of age, life expectancy for the GSD is 12 to 14 years. Thus it was anticipated that the establishment of a strong control population would be extremely difficult. In addition, limited reports had occurred (Rubinsztein *et al.*, 1996) of individuals in old-age who had remained without clinical signs of Huntington's disease despite having CAG repeat expansions in the disease size range. Another difficulty was the significant variation in normal and diseased size ranges of trinucleotide repeat expansions which had been found between laboratories.

6.3.1 Experimental Programme

A series of parallel, but distinct, approaches were instigated. The initial investigation involved the specific technique of PCR of GSD gDNA to determine the existence of the canine orthologue to *SCA1*, *CTG-B37* (DRPLA) and *MJD1* genes. This was followed by Repeat Expansion Detection (RED) analysis of CAG repeat elements *per se*, a technique which would detect trinucleotide repeat expansions without prior knowledge of the chromosomal location. At the same time Southern blotting analysis was used for the attempted identification of a specific restriction enzyme fragment harbouring the CAG expansion. As an adjunct to these

genomic manipulations, western blot analysis using the 1C2 antibody raised against the TATA-binding protein (TBP), which has specifically identified expanded polyglutamine tracts in a number of the human neurodegenerative diseases, was employed.

6.4 Canine orthologues of human genes associated with late onset progressive neurodegenerative disorders

6.4.1 Perspective

At the time of commencement of this study the molecular basis for a number of the human progressive neurodegenerative disorders had been characterised. Primer pairs, for use in PCR were available for three of the conditions, namely SCA1, DRPLA and MJD. As part of a study w3hich investigated the characterisation of the gene causing SCA1 and identification of its murine orthologue, sequence analysis had been carried out on a 3kb segment of the murine *Sca1* gene. Results indicated that *Sca1* encodes a predicted protein of 792 amino acids which showed 89% amino acid identity with the human protein. In addition, preliminary analysis of the murine locus suggested that it was similar to the human locus and had two large exons which contained the coding region and a very long 3/UTR with sequence homology which extended into the 5/UTR (85%) and 3/UTR (63%) regions (Banfi *et al.*, 1994). These findings suggested the existence of a reasonable degree of cross-species homology between the human and canine *Sca1* genes.

Could a canine orthologue to any of these three genes, namely *SCA1*, *CTG-B37* or *MJD1*, be identified by PCR ? (see Figure 21, page 171). The first step in this investigation was the random testing of the available human primers by PCR. The initial aim being to ensure that the primer pairs were not species-specific. Once it was established that the primers would amplify canine gDNA, they were tested against a number of affected dogs and an age-matched control group in an attempt to ascertain if a product similar to that produced in affected humans was seen in the dogs with CDRM.

6.4.2 Primers

Sequences for the primer pairs used to amplify genomic fragments of *SCA1* (SCA1) and *CTG-B37* (DRPLA) genes in addition to the control PCR product lengths are detailed in Table 6, page 61. The partial cDNA clone for ataxin-1 was kindly donated by Dr H. Zoghbi, Departments of Paediatrics and Molecular and Human Genetics, Baylor College of Medicine, Houston. The primer pairs available for

MJD1 (MJD) were pulled out following a data base search, but although the two available reverse primers were exonic, the only forward primer was entirely intronic (Kawaguchi *et al.*, 1994). This essentially ruled them out for use in cross-specific amplification of the canine orthologue of the *MJD1* gene.

6.4.3 Results

6.4.3.1 Rep-1/Rep-2

RT-PCR of pAx which harbours a partial cDNA ataxin-1 human clone confirmed a band around 200bp corresponding to the predicted PCR product of 214bp (Chong *et al.*, 1995). Figure 22 (page 172) indicates that this product is present in both affected and unaffected dogs but it does not occur in the mouse. A total of nine affected and two control dogs were investigated. Despite altering the Mg concentration, the addition of 2% formamide and alteration of the annealing temperature, the presence of spurious bands was invariant.

6.4.3.2 DRPLA-1/DRPLA-2

A band corresponding to the predicted PCR product size of 140bp was readily resolvable against human gDNA. PCR product obtained from mouse had no corresponding signal at 140bp, however there was a band corresponding to approximately 130bp. A total of two affected and two control dogs were tested with these primers, there was no evidence of the band of interest in any of the dogs tested Figure 23, page 173.

6.4.4 Discussion

These studies were equivocal regarding the presence of a canine orthologue to either *SCA1* or *CTG-B37*. This issue could be resolved by performing a Southern blot analysis and probing with the isolated *SCA1* or *CTG-B37* fragment respectively. This would (a) identify if a canine orthologue was present and (b) whether there was any size difference between dogs with CDRM and unaffected dogs, in each case.

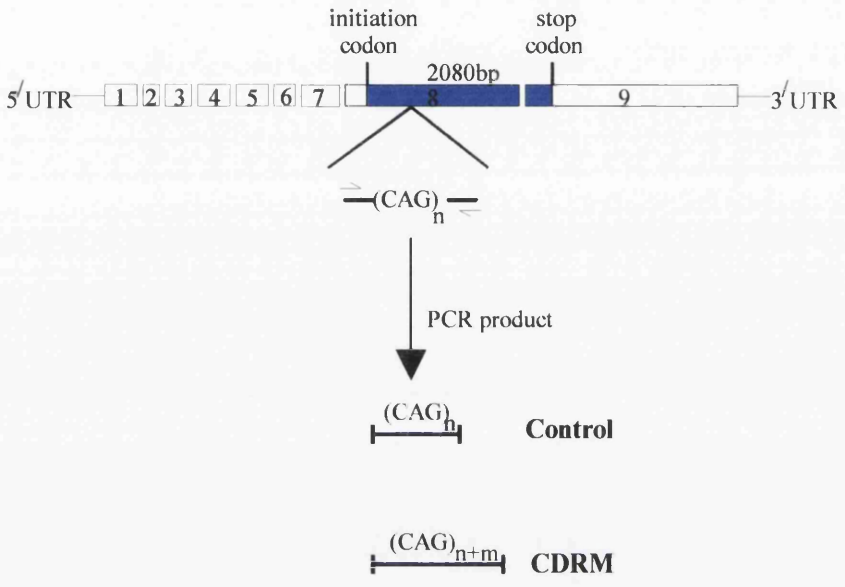


Figure 21. A schematic representation of the *SCA1* transcriptional unit illustrating the anticipated differences in PCR product profile that would be between CDRM and control dogs if there was an expansion similar to that found in SCA1 in man.

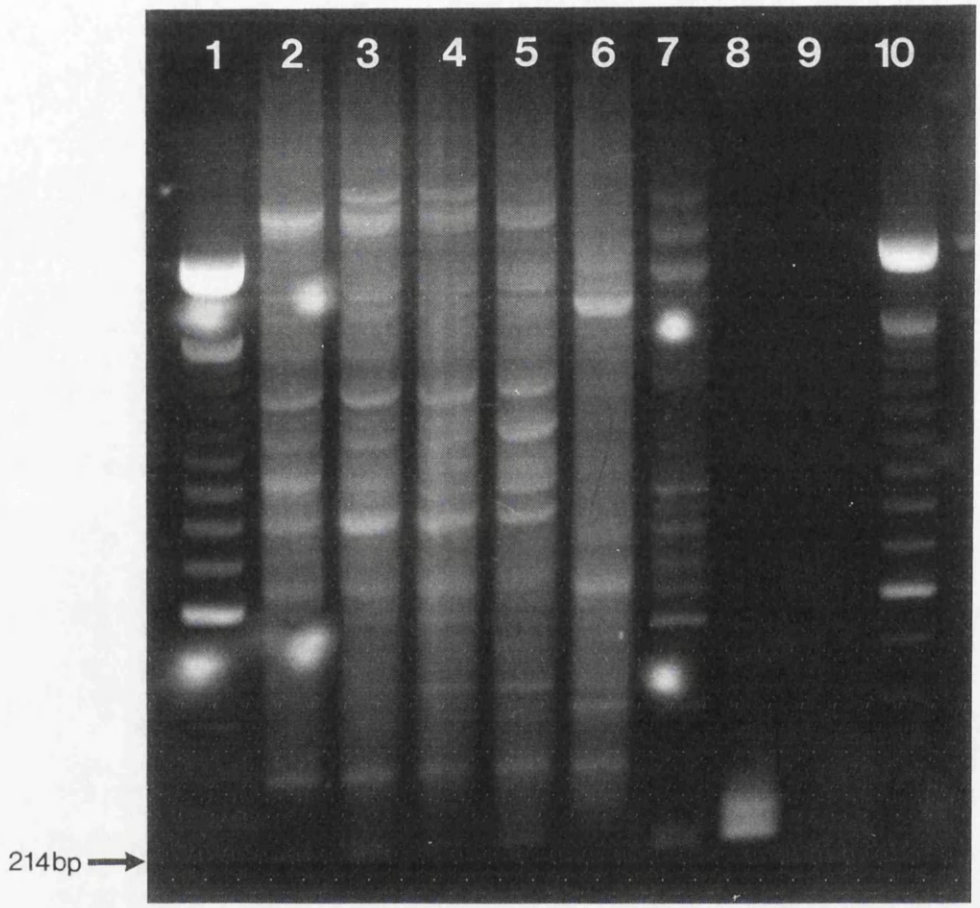


Figure 22. Products of PCR reaction using primers Rep-1 and Rep-2. Lanes 1 and 10 100bp DNA ladder, lanes 2 and 3 gDNA from two unaffected GSDs (Dogs 66 and 77), lanes 4 and 5 gDNA from two GSDs with CDRM (Dogs 49 and 3), lane 6 gDNA from normal mouse, lane 7 human gDNA, lane 8 pAx (positive control), lane 9 SDW (negative control).

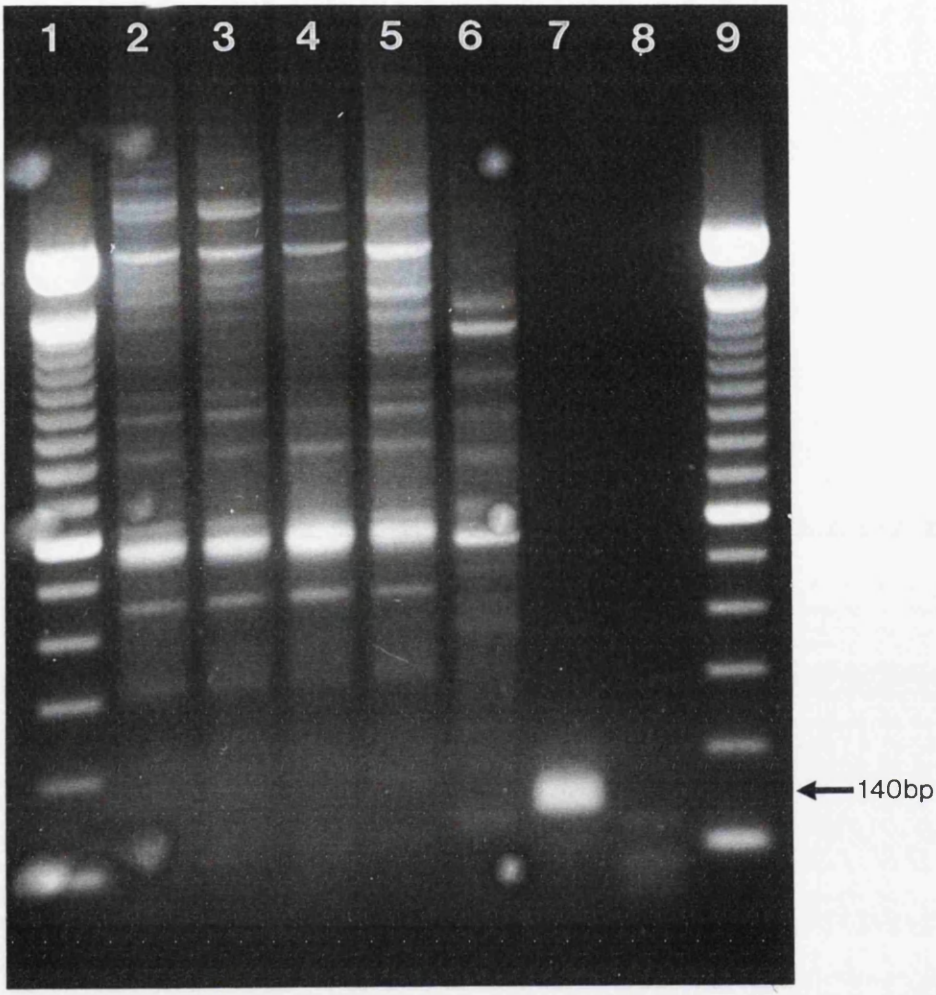


Figure 23. Products of PCR reaction using primers DRPLA-1 and DRPLA-2. Lanes 1 and 9 100bp DNA ladder, lanes 2 and 3 gDNA from two unaffected GSDs (Dogs 66 and 77), lanes 4 and 5 gDNA from two GSDs with CDRM (Dogs 3and 49), lane 6 gDNA from normal mouse, lane 7 human gDNA (positive control) and lane 8 SDW (negative control).

6.5 Southern blot analysis

6.5.1 Perspective

A preliminary data base search (data not presented) confirmed as in other mammalian genomes, CAG elements of varying unit length were well represented throughout the canine genome. The basic aim of this Southern blotting analysis study was to detect and identify small restriction enzyme fragments harbouring CAG repeats. The experimental design, as depicted in Figure 24 (page 175), involved enzymatic digestion of affected and control canine gDNA with a cocktail of four cutter restriction enzymes (Figure 25, page 176) which did not contain CAG or its complement within their recognition sequence. The high predicted frequency of four base recognition *i.e.* $1:4^4$ in combination with other four cutters, formed the basis of expectation that size differences due to variation in the CAG content within small restriction enzyme fragments would be resolvable at the level of Southern detection.

As detailed in Figure 24 (page 175) such an approach may have led to the identification of a "footprint" *i.e.* restriction fragment hybridisation profile which would distinguish affected from unaffected dogs. If such a "footprint" could be obtained, in addition to providing a preliminary diagnostic test, it would lead to a gene isolation programme.

Digests were performed as discussed in 2.8.5.3 Restriction enzyme digestions (page 62).

A number of restriction digestion enzymes were employed (Table 7, page 62). Three groups of CDRM dogs were selected for Southern blotting analysis on the basis of their classification of (a) clinical diagnosis of CDRM (Dogs 33, 32, 56, 57, 30) (b) diagnosis of CDRM confirmed at post mortem (Dogs 14, 2, 31) (c) diagnosis of CDRM confirmed at post mortem and had shown a possible expansion with the RED technique (6.6.1 Perspective, page 180) (Dogs 18, 17, 3). In addition, two groups of control dogs were selected, three non-GSDs (Dogs 101, 103, 104) and seven GSDs with no clinical signs of CDRM (Dogs 62, 74, 61, 60, 82, 68 and 69).

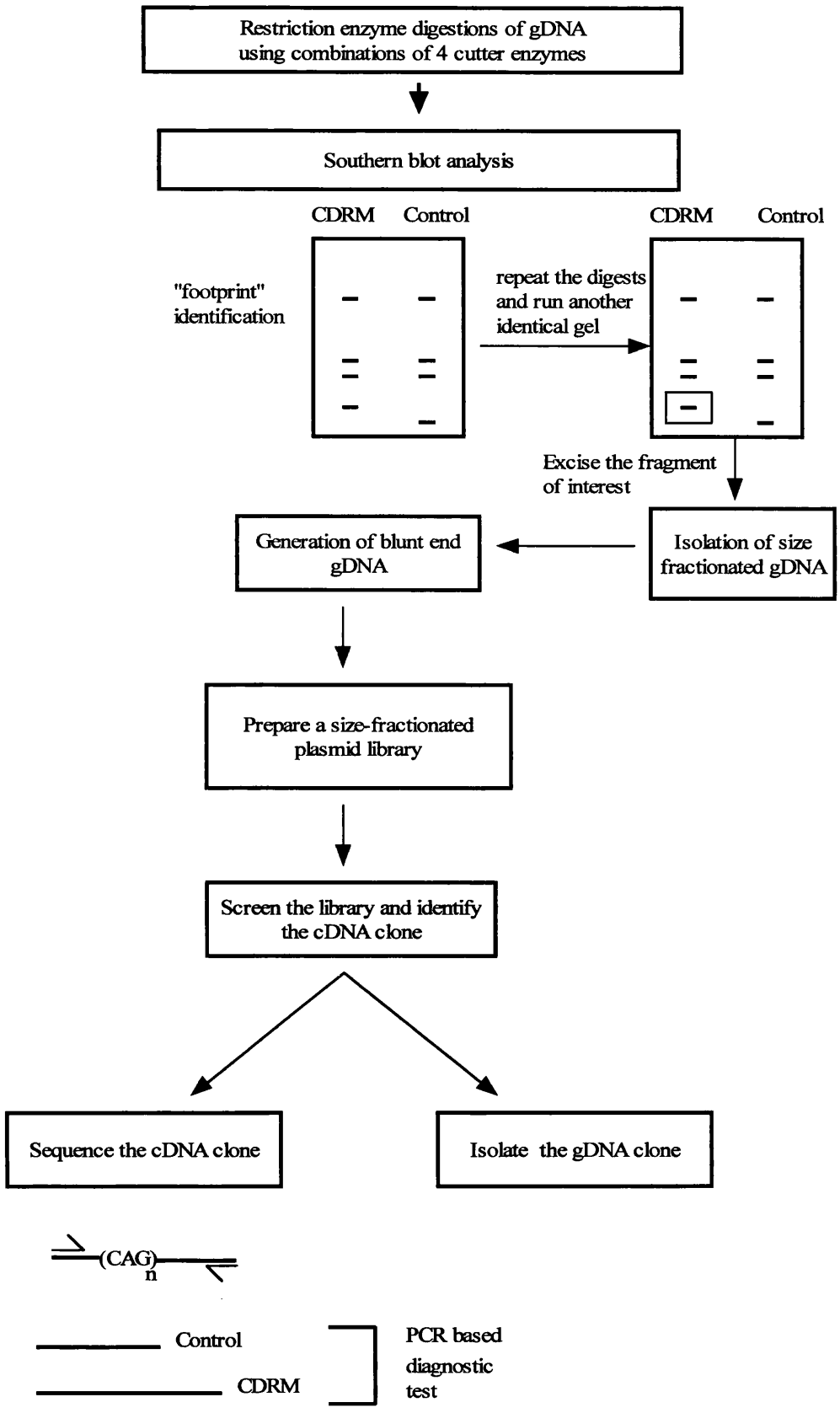


Figure 24 Overall strategy in relation to the Southern analysis if constant differences between CDRM cases and unaffected dogs were identified.

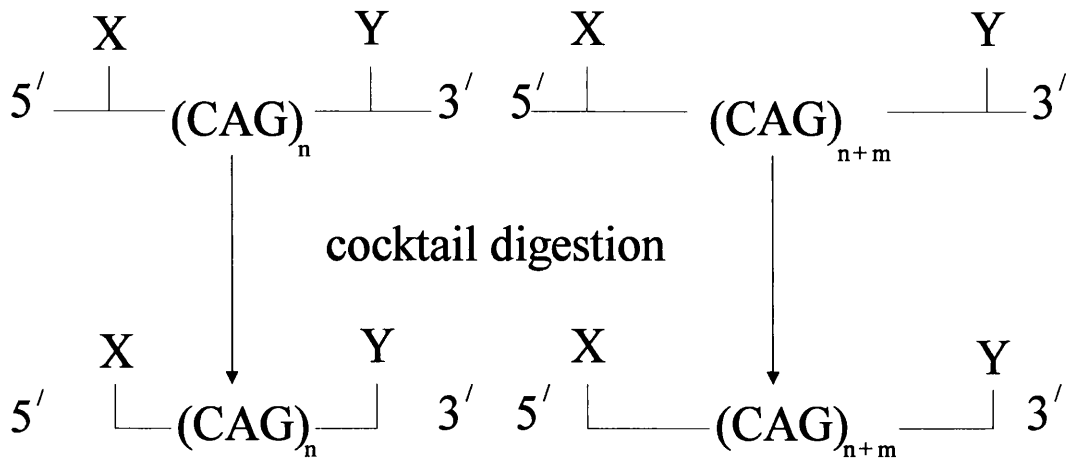


Figure 25. Schematic outline of the experimental strategy employed to size fractionate restriction enzyme fragments harbouring putative CAG expanded repeats associated with CDRM and control dogs.

6.5.2 Results

The integrity of the high molecular weight (MW) gDNA used throughout the study which remained intact following prolonged incubation at 37°C and sequential reduction in the size of the digested gDNA population following digestion with various restriction enzyme cocktails of four and six cutters is illustrated in Figure 26 (page 178).

Autoradiograph of Southern blot showing differences in hybridisation profiles using random primed ^{32}P labelled DNA probe generated from DM 200 cloned PCR product for seven affected dogs and three unaffected dogs is illustrated in Figure 27 (page 179). Other combinations of enzymes were tested (data not presented) but, as is depicted in Figure 27 differences in hybridisation profiles could not be equated with dogs affected and those unaffected by CDRM.

6.5.3 Discussion

The ambition of the Southern analysis was to determine whether it was possible to produce a diagnostic "footprint" for dogs affected with CDRM. The results obtained suggested that there were different hybridisation profiles but these could not be resolved into affected and non-affected groups. It was decided that although a more concentrated and detailed study may indeed have resolved the problem, the effort and the time could not be justified. There were several other possibilities for investigating the problem which could potentially provide a more direct route to the answers.

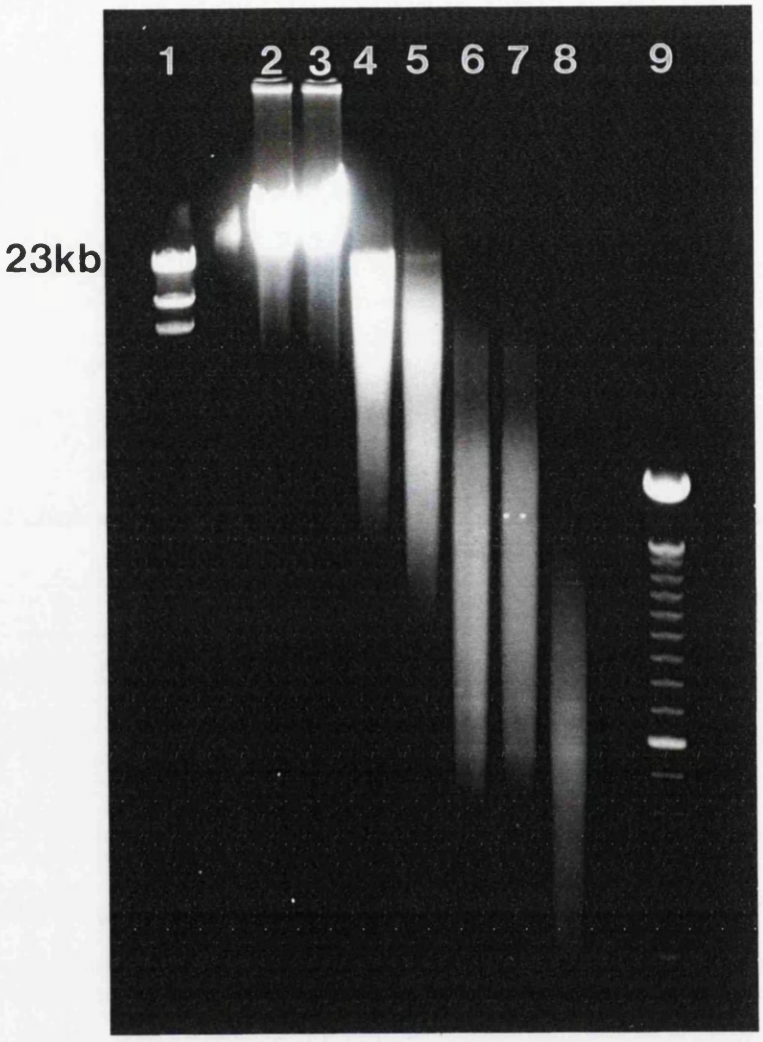


Figure 26. Example of canine gDNA undigested and digested with a range of four cutter and six cutter restriction digestion enzymes run out on a 0.7% agarose gel. The two markers used are λ *Hin*dIII (lane 1) and 100bp ladder (lane 9). Lane 2 undigested gDNA, lane 3 undigested gDNA incubated at 37°C, lane 4 gDNA digested with *Bam* HI, lane 5 gDNA digested with *Bam* HI and *Hin*d III, lane 6 gDNA digested with *Hae* III, lane 7 gDNA digested with *Hae* III and *Hha* I, lane 8 gDNA digested with *Hae* III, *Hha* I and *Sau* 3AI.

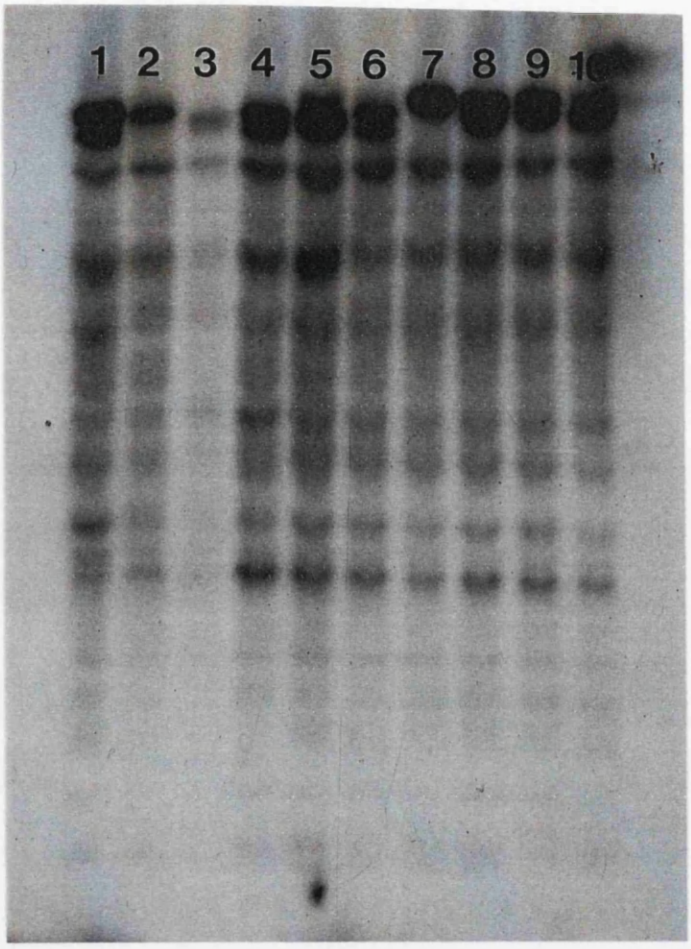


Figure 27. Autoradiograph of Southern blot showing differences in hybridisation profiles, using random primed ^{32}P labelled DNA probe generated from DM 200 cloned PCR product. The gDNA had been digested with *Bam*HI and *Hae* III. Lanes 1, 5, 6, 7, 8, 9 and 10 are GSDs with CDRM (Dogs 14, 33,32, 39, 31, 57 and 42), lanes 2, 3, and 4 are normal non-GSDs (Dogs 101, 104 and 33).

6.6 Repeat expansion detection (RED)

6.6.1 Perspective

Repeat expansion detection was a technique first developed by Schalling in 1993 (Schalling *et al.*, 1993). The RED technique was a genome-wide screening method and represented a shortcut in identifying a subtype of mutations, compared to linkage and positional cloning (Lindblad *et al.*, 1996). It therefore had the potential for identification of a pathological repeat expansion in a genetically uncharacterised disease without any knowledge of the chromosomal location of the mutation or candidate disease gene. Genomic DNA was used as template for a two-step cycling process which generated oligonucleotide multimers when expanded trinucleotide sequences were present in large numbers.

The aim was to utilise the RED technique to look for a potentially pathological repeat expansion in GSDs.

The basis of this technique was the ability of a single oligonucleotide to hybridise to its complementary strand in gDNA. A thermostable ligase was used to covalently link adjacent oligonucleotides which formed a larger single-stranded fragment. Repeated cycles of denaturation and annealing followed by ligation resulted in the formation of detectable levels of fragments which represented multimers of the oligonucleotide. The accumulation of ligation products was strongly dependent on the amount of substrate DNA, active enzyme and the cycle number used. Use of a high molar excess of oligonucleotide, ensured that any previously ligated products would be unable to further ligate, thus the maximum fragment size generated would correspond closely to the actual size of the longest repeat with a particular sequence motif in the genome. This technique is illustrated in Figure 28 (page 181). Details of the mouse gDNA which was used as an internal control are given in 2.8.6.3 Ligation (page 64).

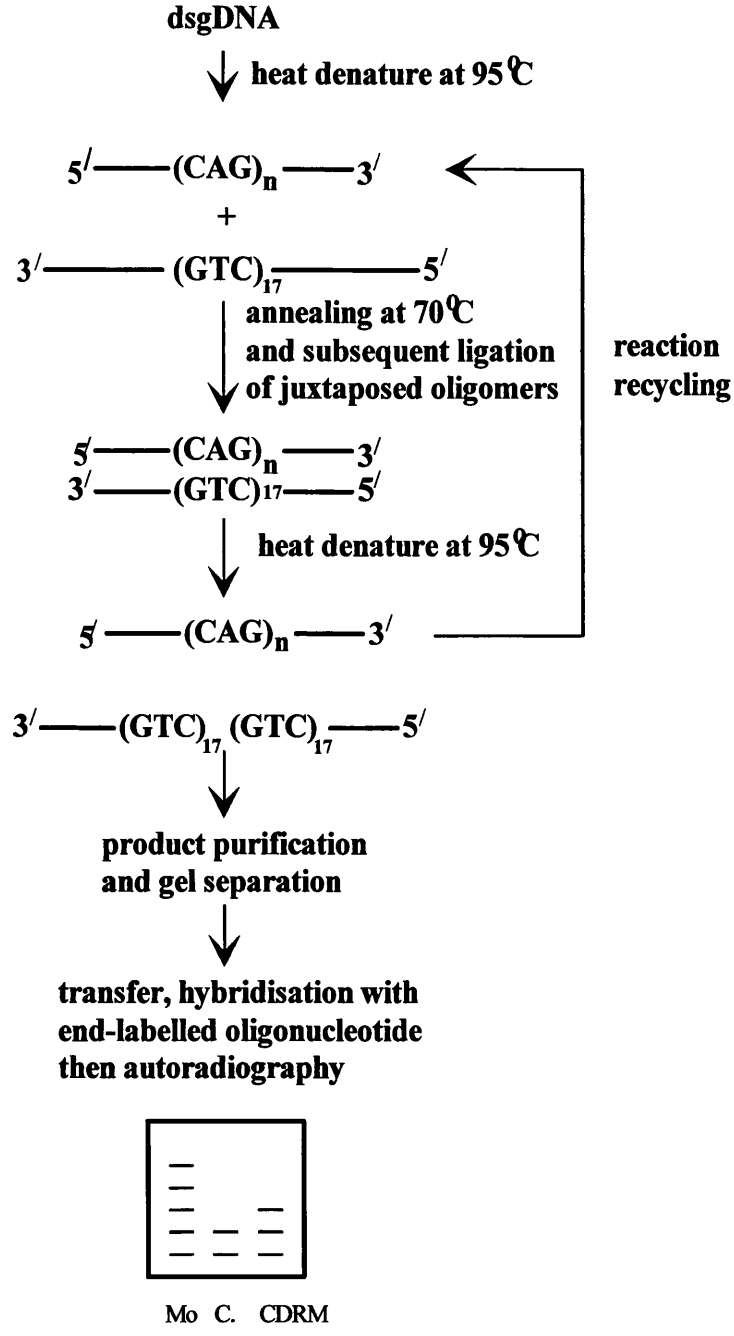


Figure 28. A schematic representation of the repeat expansion detection (RED) technique outlining the key steps. Mo mouse control, C. control dog, CDRM affected dog.

Two oligonucleotides were selected, a 51mer comprising 17 CTG repeats (Cruachem) and a 30mer comprising 10 CTG copies (Oligonucleotide Synthesis, Yale Medical School), these were within a suitable size range for the anticipated expansions and had shown consistent results when used in other laboratories. A total of 120 dogs were tested. Twenty one of the CDRM cases (including three GSDXs) had been confirmed at post mortem while 36 (including one GSDX) were clinically affected. The control group included a large number of neurologically normal dogs in addition to a smaller group of dogs with well-defined focal spinal cord lesions. In the control group there were 22 GSDs, three GSDXs, 26 non-GSDs and 12 dogs with focal spinal cord lesions. A complete list of all dogs tested is in Table 1 (pages 33-44). The ideal control group would have been equal numbers of age-matched unaffected GSDs, this was operationally difficult to achieve, thus the average age of the unaffected GSDs was less than that of the affected group. However, one of the control GSDs was 13 years old (Dog 66).

6.6.2 Results

Each individual run had to be considered on its own merits, direct comparisons were possible only if the conditions resulted in an identical mouse profile. A series of experiments which utilised the 51mer are illustrated in Figure 29 (page 184), the mouse ladder is shown in each and the number of bands is presented. In the first experiment (a) one affected dog had product at 102bp, 153bp, 204bp and 255bp (Dog 5), Figure 30 (page 185), while 11 other affected dogs had a maximum product size of 153bp. The control group had signal at 102bp (Dogs 92, 89, 91, 100, 94, 86) and 153bp (Dogs 104, 78, 87, 88, 105, 84, 106, 85). In the second experiment (b) two affected dogs had evidence of product at 102bp, 153bp and 204bp (Dogs 3 and 4), Dog 3 also had a faint product band at 255bp (Figure 31, page 186), while three affected dogs had product at 102bp only (Dogs 1, 2 and 49). The remaining four gDNAs were from control animals all of which had product at 102bp only. At this stage a number of experiments were conducted using the 30mer. Results from these suggested that two affected dogs had an expansion (Dog 18 had signal up to and including 210bp while dog 17 had signal up to and including 240bp) while 26 affected dogs had no signal greater than 120bp. All control dogs had signal no greater than 90bp or 120bp. The 51mer was then used for a final group of experiments aimed at establishing whether or not a CAG expansion was involved in CDRM.

In (c) all dog gDNAs tested had a maximum signal size of 102bp, this included four affected (Dogs 40, 6, 15, 33) and four unaffected GSDs (Dogs 66, 67, 68, 69). In (d) three affected dogs (Dogs 35, 53, 54), two old unaffected GSDs (Dogs 66 and 68) and two control dogs which were non-GSDs were tested, all had a maximum

signal of 204bp. Considering (e), (f) and (g) as a group, all dogs tested had a maximum signal of 153bp (Figure 32, page 187). This group included 12 affected dogs (Dogs 41, 42, 4, 51, 43, 44, 31, 32, 45, 48, 46, and 47), two of which were run twice on separate occasions (Dogs 4 and 45), the remainder were from GSDs of eight years and older with no clinical signs of CDRM at the time of collection of the blood sample.

gDNA was extracted from the brain (not the red nucleus as this was being collected to make a cDNA library) of eight post mortem cases, because this somatic tissue might have had a greater number of expansions than blood, so sensitivity would have been improved. Five of the dogs were CDRM cases (Dogs 28, 50, 31, 33 and 3) and three were dogs with focal spinal cord lesions (Dogs 118, 119 and 120). The aims were to examine the signal obtained with gDNA extracted from brain for these dogs, the results were then compared with those obtained from blood for the same group of dogs. Initially all eight brain gDNA samples were tested. Under these conditions the mouse control had a maximum signal of 357bp. In this run two of the affected dogs (Dogs 50 and 31) had a maximum product size of 153bp. The remaining gDNAs gave no signal above 51bp (the 51bp product had run off the bottom of the gel). This experiment was repeated, on this occasion the mouse control gave a maximum product size of 306bp, the same two dogs (Dogs 50 and 31) had a maximum signal of 102bp while two more CDRM cases (Dogs 3 and 28) had signal at 51bp. As before, none of the controls had any visible signal. A comparison between gDNA extracted from blood and brain was run for four dogs. Three affected (Dogs 28, 50 and 33) and one control (Dog 120) were tested. The mouse control had a maximum signal size of 255bp. All four gDNAs from blood samples had bands at 51bp and 102bp, while that from one of the CDRM cases (Dog 28) also had a band at 153bp. Of the four samples of gDNA from brain, one of the affected dogs (Dog 50) had a visible band at 51bp while none of the control dogs had any visible band (Figure 33, page 188).

6.6.3 Discussion

Of the 57 affected dogs tested, five were RED positive. Of the 63 control dogs tested there was no evidence for an expansion, this included the twelve animals with focal spinal cord lesions. It was technically difficult to reproduce the sensitivity of the technique, however the extent of separation of the mouse positive control was a useful guide to sensitivity in each run. Overall, the results suggest that there may be an expansion involved in a number of dogs with CDRM. There were a number of possible reasons why the results were equivocal. It may be that the technique was not sensitive enough to pick up what may be a relatively small expansion. An obvious modification would be to reduce the length of the oligonucleotides.

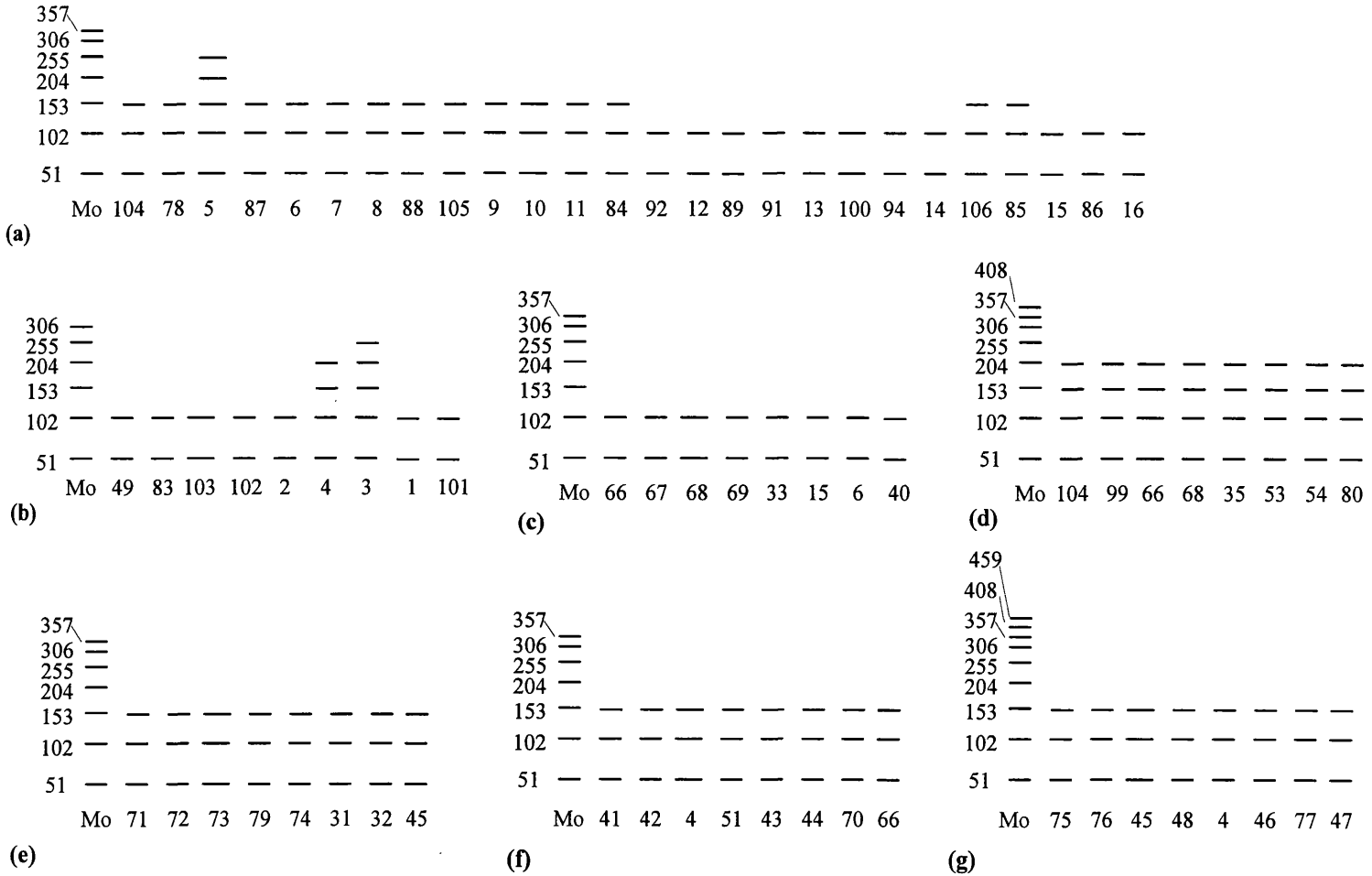


Figure 29. Schematic representation of autoradiographs produced by the repeat expansion detection (RED) technique; (a) to (g) represent seven individual experiments, these are all described in detail in the text. Mo = mouse control, numbers arranged vertically represent fragment sizes of product, numbers arranged horizontally represent dog identification numbers.

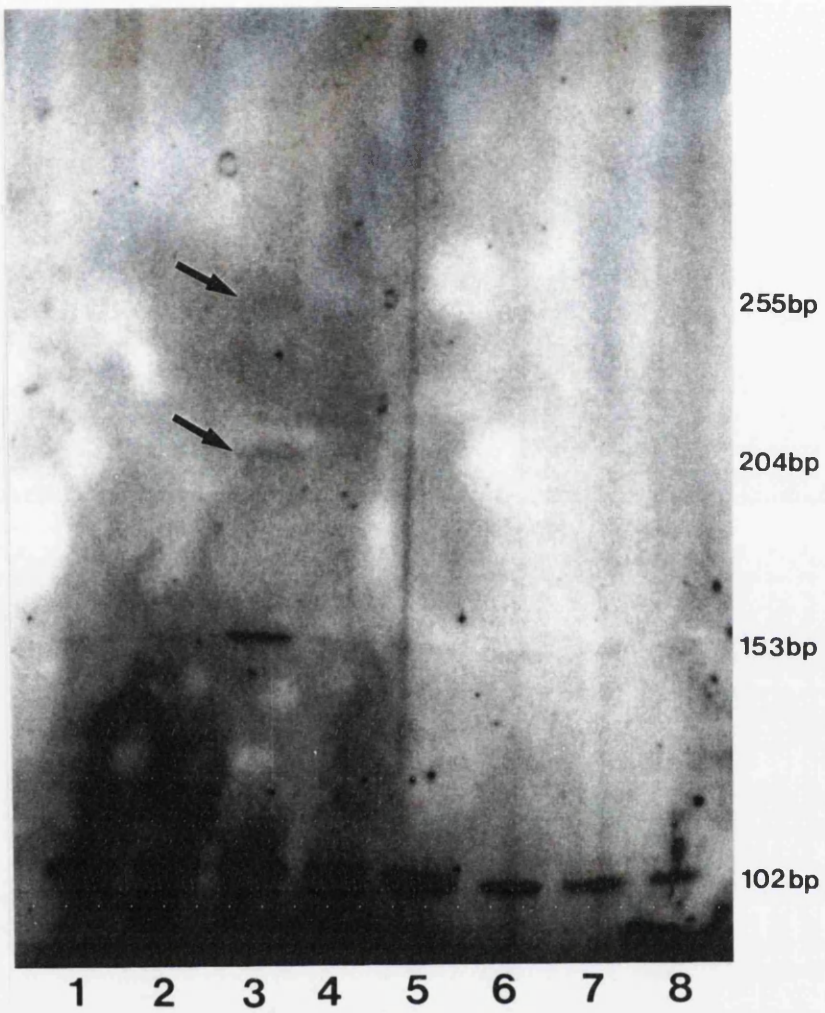


Figure 30. Autoradiograph of the RED technique using ^{32}P-labelled $(CAG)_{10}$. Lanes 1, 2, 4 and 8 normal non-GSDs (Dogs 104, 78, 87 and 88 respectively), lanes 3, 5, 6 and 7 GSDs with CDRM (Dogs 5, 6, 7 and 8 respectively). Dog 5 has product up to and including 204bp and 255bp (arrows). (Performed by Dr. Bonnie King).

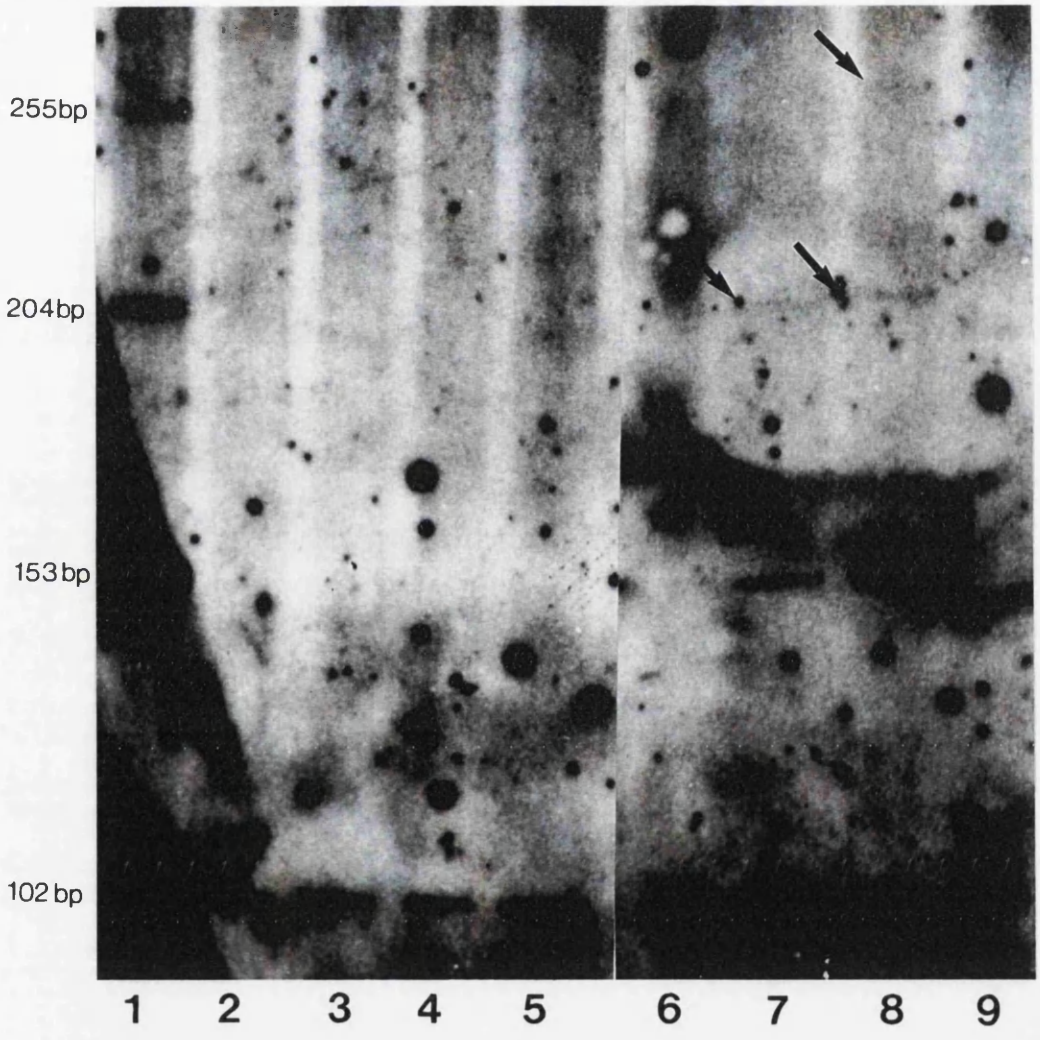


Figure 31. Autoradiograph of the RED technique using ^{32}P-labelled $(CAG)_{10}$. In all cases the gDNA was derived from blood. Lane 1 mouse ladder, lanes 2, 6, 7, 8 and 9 are from dogs with CDRM (Dogs 49, 7, 4, 3 and 1 respectively), lanes 3, 4 and 5 are from control dogs (Dogs 83, 103 and 102 respectively). Dogs 4 and 3 have product up to and including 204bp while Dog 3 had a faint band at 255bp (arrows). (Performed by Dr. Bonnie King).

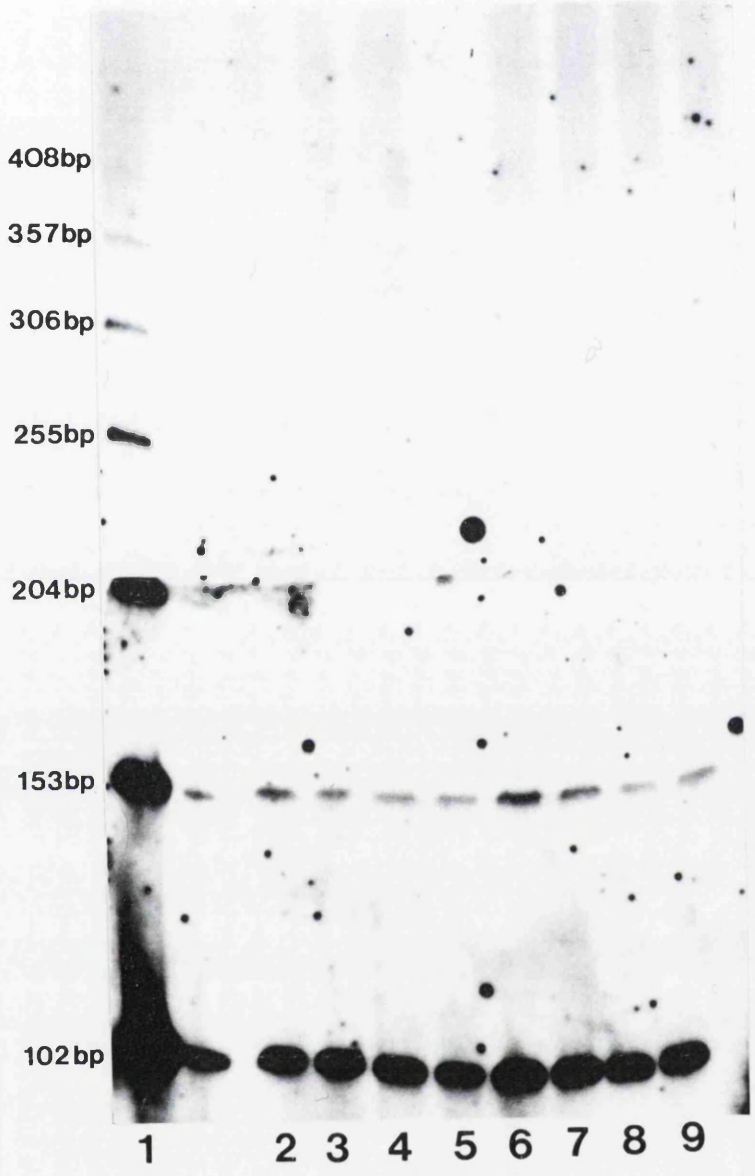


Figure 32. Autoradiograph of the RED technique using ^{32}P-labelled $(CAG)_{10}$. In all cases the gDNA was derived from blood. Lane 1 mouse ladder, lanes 2, 4, 5, 6 and 7 are from dogs with CDRM (Dogs 47, 46, 4, 48 and 45 respectively), lanes 3, 8 and 9 are from control dogs (Dogs 77, 76 and 75 respectively).

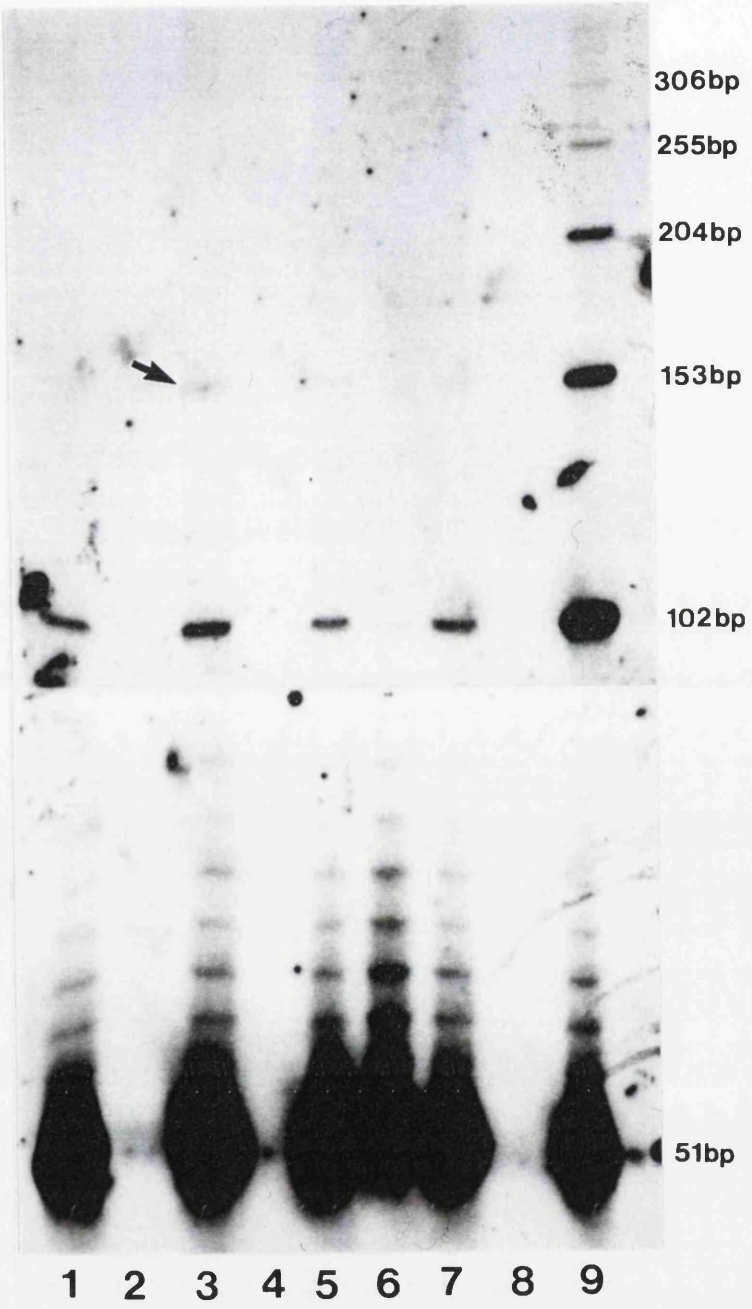


Figure 33. Autoradiograph of the RED technique using ^{32}P-labelled $(CAG)_{10}$. The gDNA is derived from two sources; blood and brain, for each dog. Lane 1 Dog 120 (control) gDNA from blood, lane 2 Dog 120 gDNA from brain, lane 3 Dog 28 (CDRM) gDNA from blood, lane 4 Dog 28 gDNA from brain, lane 5 Dog 50 (CDRM) gDNA from blood, lane 6 Dog 50 gDNA from brain, lane 7 Dog 33 (CDRM) gDNA from blood, lane 8 Dog 33 gDNA from brain, lane 9 mouse ladder. Dog 28 (gDNA from blood) gave a 153bp product (arrow).

6.7 Western blot analysis

6.7.1 Perspective

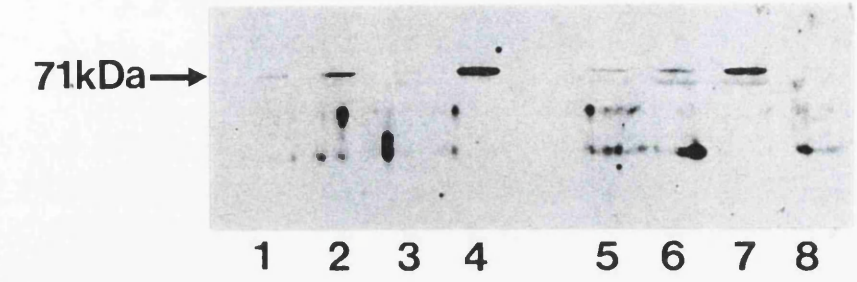
A monoclonal antibody named 1C2, had been raised against TATA-binding protein (TBP), which had a stretch of 38 polyglutamines (Trottier *et al.*, 1995). This monoclonal antibody specifically recognised peptides overlapping the polyglutamine stretch. It had been used successfully to detect expanded polyglutamine repeats in Huntington's disease, SCA1 and SCA3, when applied to lymphoblastoid cell lines (LCLs) in patients affected by these diseases. As all the dogs to be tested were to be euthanased and an abundance of suitable tissue could be collected there was no need to develop LCLs. The MOBP antibody was used as a qualitative and quantitative control (Figure 34b, page 190).

The aim was to use the 1C2 monoclonal antibody to look for pathogenically expanded polyglutamine tracts in dogs with CDRM.

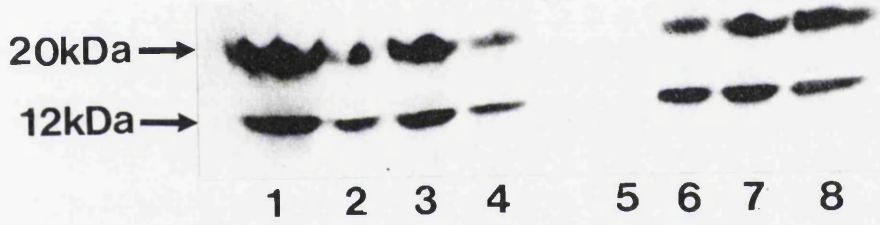
In this study the western blot analysis was carried out on protein lysates from whole brain which had been collected within 40 minutes of death and stored in liquid nitrogen, details of the preparation are given in 2.8.7.1 Sample preparation, page 66. A total of eight samples were tested, three from dogs with focal spinal cord lesions (Dogs 118, 119 and 120) and five from dogs with CDRM (Dogs 3, 28, 31, 33 and 50). The positive control was the protein extract from a SCA1 cell line (Coriell Cell Repositories, Bar Harbor), (Figure 35, page 191). Full details of the technique are given in 2.8.7 Western blot analysis (pages 66-68).

6.7.2 Results

The first run indicated a band corresponding to 71kDa for six of the dogs tested (Dogs 120, 119, 118, 28, 31,33), the remaining two dogs (Dogs 3 and 50) had no band (Figure 35, page 191). A second run with the same samples resulted in a 71kDa product in a different group of six dogs (Dogs 120, 118, 28, 50, 31, and 3) and no product in two of the dogs (Dogs 119 and 33), (Figure 34, page 190).



(a)



(b)

Figure 34. Fluorographs of western analysis on protein lysates of whole brain using the ECL technique. Lanes 1, 2 and 3 dogs with focal spinal cord lesions (Dogs 120, 118 and 119 respectively), lanes 4, 5, 6, 7 and 8 dogs with CDRM (Dogs 28, 50, 31, 3 and 33 respectively). (a) 1C2 antibody (b) MOBP antibody. (Performed by Douglas Kirkham)

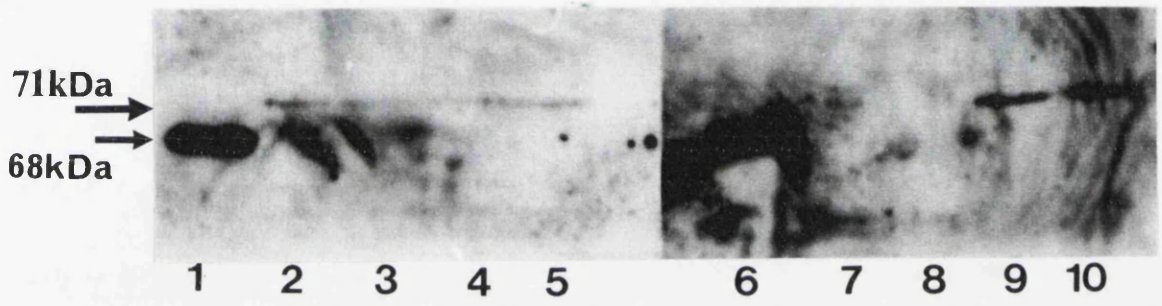


Figure 35. Fluorograph of western analysis of protein lysates from whole brain using the ECL technique. Lanes 1 and 6 SCA1 cell line (positive control) lanes 2, 3, 4, 7 and 8 dogs with CDRM (Dogs 33, 31, 28, 3 and 50 respectively), lanes 5, 9 and 10 dogs with focal spinal cord lesions (Dogs 118, 119 and 120 respectively). (Performed by Dr. Bonnie King).

6.7.3 Discussion

This preliminary set of results suggested that the 1C2 monoclonal antibody could be successfully applied to canine tissue, it also suggested that protein lysates were a suitable medium for western blot analysis with this monoclonal antibody (Figure 34, page 190). However, there was no clear distinction of western profiles between CDRM cases and control dogs. The technique requires improvement and application to a tighter control population *i.e.* aged GSDs with no clinical signs of CDRM. The dogs tested to date were cases euthanased and post-mortemed at GUVS during the course of the project.

The ability of the 1C2 monoclonal antibody to detect altered proteins depended on the length of the polyglutamine stretch. In the human disease situation, patients proteins which contained a long polyglutamine stretch (60-85 glutamines) gave a much stronger signal intensity than those with fewer polyglutamines (~40) which suggested that this was a quantitative technique (Trottier *et al.*, 1995). It may be that the length of any polyglutamine stretch in the CDRM dogs would be too short to result in an obvious difference when compared with unaffected dogs.

6.8 General Discussion

At the start of this project neurodegenerative diseases associated with a CAG trinucleotide repeat expansion had been identified in man and investigated using a number of molecular biological techniques. Amplification of specific genomic regions harbouring the CAG repeats, by PCR, involved the application of human primers directly to canine tissue, in the expectation that there would be sufficient cross-species homology to imply a canine orthologue. Results obtained, suggested that in the case of the SCA1 primers, there was cross-species homology but there was no distinction between affected and unaffected dogs tested. Application of the DRPLA primers was less successful, none of the dogs tested had the expected product. The only other primer pairs available at this stage were those for MJD. These were not tested as the forward primer was entirely intron sequence. Intronic sequences were not well conserved between species. Accordingly there was a high probability that intronic primers would vary considerably between species thus the likelihood of detecting a canine orthologue to a human gene was very low.

Southern blot analysis was employed initially to try and find a "footprint" for affected dogs, which differed from unaffected dogs. If a "footprint" could be established it would be a starting point for gene isolation as it would provide a probe to screen appropriate libraries. In addition, isolation of the specific fragment would

allow the sequence to be determined. This in turn would allow primers flanking the repeated region to be designed, such that gDNA from dogs could be screened by PCR for size differences between affected and unaffected dogs. This approach would still be applicable if the other techniques currently being tried proved unsuccessful.

Application of the RED technique to gDNA samples from a group of affected and unaffected dogs confirmed that there were a number of products of varying size which could be identified in both the CDRM and control dog populations. This technique was applied to gDNA obtained from blood and brain, that obtained from blood proved more useful. Profound differences in the distributions of longest $(CAG)_n$ repeat lengths have been found in different human populations (Sirugo *et al.*, 1997). This suggested that it was important to have accurate information on the normal variation of trinucleotide repeats of interest within the species population being studied in order to minimise population stratification artefacts. In man, such variations were best explained by random genetic drift of alleles at $(CAG)_n$-containing loci. The mutation rate at such loci must be low, as a high mutation rate would homogenise allele frequencies within a short evolutionary period, thus any evidence of random genetic drift among populations would be obscured. Thus, gross instability events were, in all probability, a rare phenomenon.

In any investigation which involved a late-onset disease in an inbred population such as the GSD, the optimal control population would be old unaffected dogs of the same breed. This was operationally difficult as GSDs could develop clinical signs of CDRM over a wide age range. To compensate for this possible problem, a large number of non-GSDs of widely varying ages (three to thirteen years old) were tested alongside the GSDs with CDRM. Results of this study would suggest that, depending on specific experimental conditions, dogs unaffected by CDRM can have repeat lengths corresponding to ligation of up to four $(CTG)_{17}$ oligonucleotides which was equivalent to genome lengths of $(CAG)_{68}$ to $(CAG)_{84}$. However, dogs in the unaffected group most commonly had ligation of up to three $(CTG)_{17}$ oligonucleotides. Dogs with CDRM can have repeat lengths corresponding to five $(CTG)_{17}$ which was equivalent to genome lengths of $(CAG)_{85}$ to $(CAG)_{101}$. Using the 10mer, the control dogs had a maximum of four ligated $(CTG)_{10}$ while the two CDRM cases which had evidence of an expansion had seven and eight ligated $(CTG)_{10}$ oligonucleotides. Taking the results as a whole, but ignoring RED run (d) in Figure 29 (page 184) which varied in sensitivity compared to other runs in the group, the maximum number of repeats found in the unaffected dogs was 51 while the affected dogs with "expansions" had a minimum of 70 trinucleotide repeats. Of the affected dogs tested by the RED technique only a very small proportion (9.4%)

had evidence for a possible trinucleotide expansion. However, none of the unaffected dogs either GSDs or other breeds had any evidence of expansion. This fact alone suggested that a trinucleotide repeat expansion cannot be excluded as a possible factor in the development of CDRM.

These findings suggested that either CDRM was caused by a trinucleotide repeat expansion in combination with other factors, or the expansion was too small to be detected by the size of oligonucleotides used. The use of smaller oligonucleotides would improve the sensitivity of the technique and so address this question. One problem was the lack of consistency in the results. One case (Dog 4) had a product corresponding to ligation of five $(CTG)_{17}$ in one run only, despite there being better separation of the mouse ladder on subsequent runs where the maximum product for the same dog was three $(CTG)_{17}$. Recent work by Sirugo *et al* (1997) established the consistency of results with the RED technique when it was applied to both humans and non-human primates. The inconsistencies found in this study may have been another reflection of the need for more sensitive techniques in the dog compared with humans and non-human primates.

The preliminary work carried out so far on Western blot analysis using the 1C2 antibody suggested that the technique may still be useful but some fine-tuning was required. The target proteins which were defective in the human neurodegenerative diseases were dissimilar, but for the presence of polyglutamine stretches. In addition, the proteins probably had different, but as yet unknown, functions. SBMA was the only condition where the protein function was known, this protein was an androgen receptor. The 1C2 antibody had a strong length affinity, which suggested that a unique conformation was recognised which required a minimum length of polyglutamine, this conformation was then stabilised by any further increase in size (Trottier *et al.*, 1995).

In summary, the results from the above experiments provided some evidence that CDRM may be caused by a trinucleotide repeat expansion. However further work would be required to definitively prove whether or not this was the case.

7. Conclusions

The material presented in this thesis concerned the clinical presentation, neuropathology and the assessment of potential aetiologies of chronic degenerative radiculomyelopathy in the German shepherd dog. The clinical study confirmed most of the previous findings (Averill, 1973; Griffiths and Duncan, 1975a; Braund and Vandevelde, 1978; Clemmons, 1989). The only notable difference found was a clinical course of six to 18 months rather than the six to 12 months previously reported (Clemmons, 1992). This clinical course related to the time period from onset of clinical signs to loss of effective pelvic limb function. Only two cases developed thoracic limb signs which were manifested as stumbling. Thoracic limb involvement occurred approximately two years after the onset of pelvic limb signs, which had necessitated the use of "dog carts" to maintain some mobility. Clemmons had suggested, without indicating a time scale, that dogs may eventually progress to develop brain stem signs (1992). There was no indication of brain stem signs occurring in any of the dogs in the present study, even in the two cases maintained for over two years. It was unlikely that any of the cases seen in this study could have meaningfully been maintained any longer as they were very severely debilitated. Further findings from the clinical study suggested that although CDRM did occur in other large breeds of dog, it was predominantly a disease of the GSD. The finding of several pairs of affected littermates, and evidence of cases in several generations in the same pedigree lineage within the GSD breed strongly suggested that the disease had a genetic basis. A molecular investigation of this aspect of the aetiology of CDRM was instigated, and pedigree collection to allow the establishment of a GSD pedigree database is currently underway.

The neuropathology study confirmed earlier descriptions of spinal cord pathology. Lesions in the spinal cord white matter were indicative of breakdown of both axons and associated myelin sheaths, often with a concurrent microglial and/or macrophage response and astrocytosis. These changes, typical of Wallerian-type degeneration, were found in both ascending and descending tracts. The corticorubrospinal tracts (*i.e.* the overlapping corticospinal and rubrospinal tracts in the lateral funiculi) were the most severely affected, particularly from the mid-thoracic to mid-lumbar segments. There may be some evidence to suggest that the disease affects the distal portions of certain long tracts such as the corticorubrospinal tract and the fasciculus gracilis. As the rubrospinal tract is a very important motor tract in the dog, this finding was consistent with the motor deficits found on clinical examination. Comparison of gait score at death with the pattern of degeneration on histopathological examination of the spinal cord for the 25 dogs included in the pathology study suggested that there was some correlation between

the clinical signs and extent of degeneration. However, the exact pattern of degeneration could not be predicted by gait score alone. The proprioceptive deficits which occur would be consistent with the pathology seen in the major proprioceptive tracts of the spinal cord such as the fasciculus gracilis, with some involvement of the degenerated spinocerebellar tracts.

Changes were also found in specific nuclei and white matter structures of the brain. The red nucleus was the most severely and consistently affected structure. Changes in this nucleus were degenerating neurones, manifested as both neurones with eccentric nuclei and chromatolytic neurones. In addition, there was evidence of a neuronal dropout in the most severely affected dogs. The red nucleus is of great importance both as the major motor centre in animals and as a relay point for proprioceptive impulses from cerebellum to cerebral cortex (Jenkins, 1978). Thus, some of the major clinical signs seen in cases of CDRM *i.e.* ataxia and paresis were consistent with degeneration in this nucleus. The associated degeneration found in the ventral tegmental decussation and in the rubrospinal tracts provide further evidence that the changes within the red nucleus were likely to be of significance. Though the neuropathology study did not suggest a specific aetiology it has allowed some of the previously-suggested causes to be excluded. Several authors had suggested that CDRM was a chronic demyelinating disorder (Waxman *et al.*, 1980a; Waxman *et al.*, 1980b). However, both light microscopy and electron microscopy studies completed in this project strongly suggested that both axons and the myelin sheaths were involved in the degeneration thus CDRM was a neurodegeneration rather than a demyelinating disorder.

Despite the finding of previously unreported degeneration in specific nuclei and white matter areas in the brains of dogs affected by CDRM, the primary site of degeneration *i.e.* neuronal cell body versus distal axon has not been established. Examination of tissue from very early cases of CDRM would help answer this question but such material is very difficult to obtain within the boundaries of a project based on clinical cases.

There had been suggestions that vitamin E deficiency may have been involved in the aetiology of CDRM (Williams *et al.*, 1984; Williams *et al.*, 1985); however, this was highly unlikely for the dogs included in this study. Measurements of serum vitamin E indicated that normal GSDs have levels comparable with other breeds. Also, although GSDs with CDRM showed a wide fluctuation in vitamin E concentrations there was no indication of a reduction compared with either unaffected GSDs or unaffected dogs of other breeds. In this study, CDRM seemed to be concurrent with, if anything, a slightly elevated serum vitamin E

concentration. Additional evidence that affected dogs do not have a vitamin E deficiency was provided by visual comparison of the rates of degeneration for those CDRM cases given oral supplementation with vitamin E (along with vitamin B and EPO) with those given no therapy, where no obvious difference was found.

The results obtained from the various molecular techniques, which addressed the issue of whether or not CDRM was the result of a CAG trinucleotide repeat expansion, were equivocal. There were a number of possible explanations for this. The most likely explanation was that if CDRM was caused by a trinucleotide repeat expansion, then the expansion could be relatively small. The expansion may well be small in affected dogs for a number of reasons. Firstly, in other species *e.g.* the human neurodegenerative conditions caused by expanded CAG repeats, the severity of disease increased and the age of onset decreased with increasing length of expansion as often occurred between generations. Evidence from related affected dogs with CDRM does not indicate a dramatically more severe phenotype nor a significantly younger age of onset in subsequent generations. Secondly, in the human neurodegenerative diseases signs of systemic disease outwith the CNS were occasionally seen, whereas dogs with CDRM had a phenotype which appeared to affect only the CNS, which suggested that the underlying lesion may be less severe in the affected dogs. In addition, the techniques used to detect the expansions in human neurodegenerative disorders may not have been sensitive enough to pick up a small expansion in the dog genome. This does not rule out trinucleotide repeat expansion as a possible cause for CDRM, nor does it rule out the possibility that CDRM is caused by a CAG repeat expansion.

Until recently it had been assumed that greater than 40 CAG repeats represented a pathological expansion. Recent work which involved the RED technique suggested that this premise may be incorrect (Nakamoto *et al.*, 1997). In the populations tested (75 normal, healthy Japanese students and 30 randomly chosen healthy Caucasians) the incidence of more than 51 CAG/CTG repeats in normal Japanese was 42.7% while in the Caucasians it was 13.3%. In both these normal populations, CAG/CTG repeats of up to 91 were found. Work by Sirugo *et al* (1997) using the RED technique to do total genome scans, found a wide range in the size of maximum CAG/CTG repeats in the normal population, with the greatest variation found in East Asian individuals (up to 92 repeats in normal individuals). In addition, these repeat lengths at the loci being detected were relatively stable so could not be described as "expansions" with any sense of abnormality or dynamic change in length. These authors suggested that the intensity of signal may reflect the number of copies per genome, of sequences of the various longer lengths *e.g.* a locus with a $(CTG)_{115}$ should more often allow the final ligations of six $(CTG)_{17}$

than would a locus with a $(CTG)_{102}$. This suggested that if CDRM was the result of a small CAG expansion then the intensity of signal could be faint, or even not visible, dependent on the maximum CAG length present.

7.1.1 In summary

CDRM is a chronic progressive neurodegenerative disease which affects large breed dogs, with a marked predilection for the GSD. Overt clinical signs and pathology are limited to the CNS, the neuronal population of the red nucleus is specifically affected. There is some, inconclusive, evidence that a trinucleotide repeat expansion may be involved.

7.2 Future aims

A more detailed pathological description of CDRM has been established. The classification of CDRM as a chronic, progressive neurodegenerative condition has been confirmed. Future work on the pathology would involve the use of new antibodies to specific cell structures of interest, as they become available. A more detailed electron microscopy study of the red nucleus in dogs with CDRM is justified; in particular, a specific examination of the nuclei of the neurones within the red nucleus. This would be of interest as neuronal intranuclear inclusions have recently been reported in Huntington's disease, one of the chronic progressive neurodegenerative diseases in man which is characterised by a CAG trinucleotide expansion (Scherzinger *et al.*, 1997).

The construction of a data base to provide a more detailed evaluation of pedigrees to allow conventional genetic analysis of the disease is currently at the planning stages. This approach is of particular relevance due to the finding, in the course of this project, of several groups of closely-related affected GSDs.

A more direct approach to the molecular genetic background to the disease should determine whether or not expanded CAG repeats are involved in CDRM. The PCR work carried out so far was addressing the direct questions; are there canine orthologues for *SCA1* and *CTG-B37* respectively and are either of them expanded in cases of CDRM ? The results to date are equivocal so the approach would be to employ Southern transfer of the PCR products from primers used to amplify canine orthologues, followed by probing with human ataxin-1 and human atrophin-1 for which sequences have been determined. On the basis that cross hybridisation would occur, as would be expected from the degree of species homology present, this would confidently identify if canine orthologues of these genes existed. These

genes could then be compared in affected and unaffected dogs and any differences assessed.

A gene hunt would involve the construction of cDNA libraries from RNA extracted from the brain nuclei of affected and age-matched control dogs. These could be probed for the presence of expressed sequence tags (ESTs) harbouring CAG repeats. Candidate ESTs would then be isolated, sequenced and compared to data in appropriate gene banks. In parallel, given the possibility that the lesion is not a CAG expansion, a more general gene hunt would also be activated. This would involve subtractive hybridisation using the cDNA libraries already constructed. Differential display would also be employed. Upon verification by northern analysis, clones would be sequenced and compared to databanks. This technique has the advantage that it would lead directly to gene cloning. Both subtractive hybridisation and differential display technologies would determine which clones, if any, are disproportionately represented in the samples from dogs with CDRM compared with control dogs. Any clones disproportionately represented in the disease tissue could then be sequenced and compared to data banks. In addition, preparing a cDNA clone from a spinal cord injury dog as a control would confirm the specificity of any clones identified. The identification of cDNA clones, whether CAG expansions or not, would allow the preparation of genomic clones. The genomic clones in turn, would identify the molecular anatomy of the genetic lesion causing CDRM.

In conclusion, location of the affected gene in CDRM would result in the production of a diagnostic test. If a trinucleotide repeat expansion was confirmed then a straight-forward PCR based diagnostic test would be developed. If a gene was identified which did not fall into the category of a trinucleotide repeat expansion then a more complex sequence-based diagnostic test would be performed. For example, a Southern-based assay. In turn, there would be the possibility of developing a rational therapy to treat affected animals. A further advantage of cloning the putative causal gene would be to use the information gained in CDRM research to further knowledge of any similar human disorders.

8. Appendix

8.1 Tissue fixation and processing

8.1.1 Fixatives

8.1.1.1 Buffered neutral formaldehyde, 4% (BNF)

To prepare 1 litre of fixative:

40% formaldehyde*	100mls
tap water	900mls
sodium dihydrogen orthophosphate	4g
dipotassium hydrogenortho phosphate	8g

*40g paraformaldehyde in 100mls water, heat to between 60°C and 70°C add 1M sodium hydroxide drop by drop until the precipitate has cleared. Cool to 4°C.

8.1.1.2 Karnovsky's modified fixative (paraformaldehyde/glutaraldehyde 4%/5%)

To prepare 1 litre of "strong fix":

8% paraformaldehyde*	500ml
25% glutaraldehyde (EM grade)	200ml
0.08M isotonic cacodylate buffer**	to total volume 1litre
calcium chloride	500mg

Filter. Store for a maximum of 14 days at 4°C.

*8% paraformaldehyde:

40g paraformaldehyde in 500ml distilled water, heat to 65°C and clear with 1M NaOH, cool to 4°C.

**0.08M sodium cacodylate buffer:

17.1224g sodium cacodylate made up to litre with distilled water. Use 1M HCl to adjust pH to 7.2.

8.1.2 Tissue processing protocols

8.1.2.1 Resin processing

Samples were processed for araldite resin embedding using a Lynx *el* microscopy tissue processor (Leica). Processing involved the following solutions:

1)	isotonic cacodylate buffer	4°C	50 mins
2)	1% osmium tetroxide in cacodylate buffer	room temperature	2 hrs
3)	isotonic cacodylate buffer	room temperature	30 mins
4)	50% ethanol	4°C	5 mins
5)	50% ethanol	4°C	10 mins
6)	70% ethanol	4°C	5 mins
7)	70% ethanol	4°C	10 mins
8)	80% ethanol	4°C	5 mins
9)	80% ethanol	4°C	10 mins
10)	90% ethanol	4°C	5 mins
11)	90% ethanol	4°C	10 mins
12)	ethanol	4°C	20 mins
13)	ethanol	4°C	20 mins
14)	propylene oxide	room temperature	15 mins
15)	propylene oxide	room temperature	15 mins

16)	1:3 resin*:propylene oxide	room temperature	13 hrs
17)	1:2 resin:propylene oxide	room temperature	6 hrs
18)	1:2 resin:propylene oxide	room temperature	18 hrs
19)	resin	30°C	4 hrs

* resin composition see page 205.

Processed samples were embedded in resin filled silicone moulds and left to polymerise overnight at 60°C.

8.1.2.2 Paraffin wax processing

Solutions used in the Shandon Elliot 24 hour automatic tissue processor (Histokinette) to prepare spinal cords and body tissues for paraffin blocks:

1)	70% methylated spirit / 5% phenol	2 hrs
2)	90% methylated spirit / 5% phenol	2 hrs
3)	methylated spirit	2 hrs
4)	ethanol / 5% phenol	2 hrs
5)	ethanol / 5% phenol	1 hr
6)	ethanol / 5% phenol	1 hr
7)	1% celloidin in methyl benzoate*	4 hrs
8)	xylene	1 hr (x3)
9)	paraffin wax	4-6 hrs
10)	paraffin wax	7 hrs

Solutions used in the Shandon Elliot 7 day automatic tissue processor (Histokinette) to prepare brains for paraffin blocks:

1)	70% methylated spirit/5% phenol	6 hrs
2)	methylated spirit	10 hrs
3)	absolute alcohol/5% phenol	6 hrs
4)	absolute alcohol/5% phenol	6 hrs
5)	absolute alcohol/amyl acetate	4 hrs
6)	amyl acetate I	10 hrs
7)	amyl acetate II	10 hrs
8)	1% celloidin in methyl benzoate*	20 hrs
9)	1% celloidin in methyl benzoate	20 hrs
10)	xylene	4 hrs
11)	paraffin wax	14 hrs
12)	paraffin wax	10-20 hrs

*celloidin was obtained as Necoloidine (Merck) as an 8% solution which following dilution with methyl benzoate was considered as a 1% solution.

8.1.3 Mounting media

8.1.3.1 Araldite Resin

araldite CY212	(resin)	30g
dodecanyl succinic anhydride(DDSA)	(hardener)	25.2g
2,4,6-tri-dimethylamino-methylphenol(DMP 30)	(accelerator)	1.2ml
di-butyl phthalate	(plasticiser)	1.0ml

8.2 Staining protocols and stains

8.2.1 Staining of tissues for light microscopy

8.2.1.1 Haematoxylin and eosin

Sections were passed through the following solutions:

1)	xylene	2 min
2)	absolute alcohol	2 min
3)	methylated spirit	2 min
4)	water	2 min
5)	Lugols iodine (page 209)	1 min
6)	water	1 min
7)	5% sodium thiosulphate (Hypo)	1 min
8)	water	
9)	Mayer's haematoxylin (page 210)	10 mins
10)	1% acid alcohol (page 210)	3 dips
11)	water	2 mins
12)	Scots tap water substitute (page 210)	1 min
13)	water	2 mins
14)	methylated spirit	10 secs
15	saturated alcoholic eosin	2 mins
16)	methylated spirit	2 mins
17)	absolute alcohol	2 mins
18)	histoclear	2 mins

19)	xylene	5 mins

Sections were mounted in DPX permanent mounting media.

8.2.1.2 Haematoxylin

Immunocytochemistry sections were counterstained as appropriate:

1)	water	2 mins
2)	Mayer`s haematoxylin	50 secs
3)	water	wash off excess haematoxylin
4)	Scots tap water substitute	30 secs

Sections were dehydrated as above.

8.2.1.3 Cresyl Violet

Sections were passed through the following solutions:

1)	xylene	2 min
2)	absolute alcohol	2 min
3)	methylated spirit	2 min
4)	water	2 min
5)	0.1% acidified cresyl violet*	6 min
6)	acetic acid acidified meth. spirit to differentiate	1 min
7)	methylated spirit	briefly
8)	absolute alcohol	2 min
9)	histoclear	2 min
10)	xylene	5 min

*cresyl violet is acidified by the addition of 5 drops of 10% acetic acid for every 30 mls of stain, it is then pre-heated to 56°C before use.

8.2.1.4 Congo Red

1)	xylene	2min
2)	absolute alcohol	2min
3)	methylated spirit	2min
4)	water	2min
5)	haemalum	5min
6)	water	1min
7)	1% acid alcohol (page 210)	3 dips
8)	congo red	20min
9)	lithium carbonate	15sec
10)	methylated spirit	2min
11)	absolute alcohol	2min
12)	histoclear	2min
13)	xylene	5min

8.2.1.5 3,4,3`4` tetraaminobiphenylhydrochloride (DAB)

50mg	DAB
50ml	distilled water
40ml	0.2M Na_2HPO_4
add until pH is 7.3	0.2M KH_2PO_4

Filter. Use soon after preparing as light sensitive.

8.2.1.6 **Vectastain *Elite* ABC reagent**

5 mls	PBS
2 drops	reagent A
2 drops	reagent B

Mix well, stand for 30 minutes before use.

8.2.1.7 Mountant for immunofluorescence

Citifluor (Chemlab, University of Kent)

8.2.2 Staining of tissues for electron microscopy

1)	saturated uranyl acetate in 50% ethanol	5-15 mins
2)	50% ethanol	rinse
3)	50% ethanol	rinse
4)	distilled water	rinse x 2
5)	air dry	
6)	Reynold`s lead citrate* (Sodium hydroxide moistened chamber)	5-10 mins
7)	1M sodium hydroxide	rinse x 3
8)	distilled water	rinse x 5

*details 8.2.2.1.6 Reynold`s lead citrate, page 210.

8.2.2.1.1 Lugols Iodine

1g	Iodine
2g	Potassium iodide

Made up to 100mls with distilled water.

8.2.2.1.2 Mayer`s Haematoxylin

1.0g	haematoxylin
10.0g	potassium alum
0.2g	sodium iodate

Made up to 1 litre with distilled water. Bring to boiling point, allow to cool overnight then add:

1.0g	citric acid
50g	chloral hydrate

8.2.2.1.3 1% Acid Alcohol

1% HCl in methylated spirit

8.2.2.1.4 Scots tap water substitute:

3.5g	sodium bicarbonate
20.0g	magnesium sulphate

Made up to 1 litre with distilled water

8.2.2.1.5 Methylene blue/azur II:

1%	methylene blue
1%	azur II
1%	borax

In distilled water

8.2.2.1.6 Reynold`s lead citrate

1.33g	lead nitrate
1.76g	sodium citrate

Dissolve each in 15ml distilled water, mix the two together and shake vigorously for 1 minute followed by occasional shaking for the next 30 minutes. Clear with 8.0ml

1M sodium hydroxide, make up to final volume with 50ml distilled water (final pH12).

8.3 Buffers

8.3.1 General buffers

8.3.1.1 Phosphate buffer

~80% Na_2PO_4

~20% KH_2PO_4 added to pH 7.4

8.3.1.2 Phosphate buffered saline (PBS)

8.006g	NaCl
0.2012g	KCl
0.2042g	KH_2PO_4
1.1356g	Na2PO4 (pH 7.3)

In 1 litre distilled, deionized water. Sterilise by autoclaving.

8.3.1.3 Tris buffered saline (TBS)

25mM Tris pH 7.5

136mM sodium chloride

2.6mM potassium chloride

8.3.1.4 Tris EDTA (TE) buffer

10mM tris pH 8, 1mM EDTA

500µl	1M tris pH 8
100µl	0.5M EDTA

8.3.2 Agarose gel running buffer

8.3.2.1 Tris acetate EDTA buffer (TAE)x10

0.04M Tris acetate, 0.001M EDTA

48.4g	tris base
11.4ml	glacial acetic acid
20ml	0.5M EDTA (pH 8)

made up to 1 litre in distilled water, final pH 7.6

8.3.3 SDS-Page gel buffers for RED

8.3.3.1 Tris borate EDTA buffer (TBE)x10

108g	Tris base
55g	boric acid
40ml	0.5M EDTA (pH 8)

made up to 1 litre in distilled water, final pH 8.2-8.4

8.3.4 SDS-Page gel buffers for westerns

8.3.4.1 Stacking gel buffer

0.5M Tris HCl pH 6.8

6g	Tris pH 6.8
40ml	distilled water
48ml	1M HCl

Made up to 100mls with distilled water.

8.3.4.2 Resolving gel buffer

3.0M Tris HCl pH 8.8

36.3g	Tris
48ml	HCl 1M

Made up to 100ml with distilled water.

8.3.4.3 Reservoir bufferx10

30.3g	Tris
144g	glycine
10g	SDS

Made up to 1 litre with distilled water.

8.3.5 Tris buffered saline (TBS)

2.4g	Trizma base
8.0g	sodium chloride

made up to 1 litre with distilled water, adjust pH to 7.6 with hydrochloric acid. Store at 4°C.

8.3.6 Tris buffered saline-Tween (TBS-T)

Dilute required volume of Tween™-20 in TBS (see above) to give a 0.1%(v/v) solution. Store at 4°C.

8.3.7 Sodium chloride/sodium citrate (SSC) x 20

3M sodium chloride/0.3M sodium citrate

1753g	sodium chloride
882g	sodium citrate

made up to 10 litres with distilled water

8.4 Loading dyes

8.4.1 6 x buffer for TAE conditions

30% glycerol (Sigma)

0.25% bromophenol blue

0.25% xylene cyanol FF (Sigma)

8.4.2 5 x stop solution for TBE conditions (RED)

95% formamide

20mM EDTA

0.05% bromophenol blue

0.05% xylene cyanol

8.4.3 SDS-Page loading buffer for westerns

0.0625M Tris HCl pH 6.8

2% SDS

10% glycerol

Only add the following as buffer is being added to samples:

0.002% bromophenol blue

5% mercaptoethanol/40mM DTT (dithiothreitol)

8.5 Reagents for genomic DNA isolation

8.5.1 RNase A

To prepare a DNase-free stock solution:

1) Dissolve RNase A to the required concentration in 10mM Tris-HCl pH 7.5, 15mM NaCl.

2) Heat to 100°C for 15 minutes.

3) Allow to cool to room temperature. Store in aliquots at -20°C.

8.5.2 Proteinase K

Proteinase K was supplied lyophilised with the QUIAmp® kit. It was reconstituted in SDW at 18mg/ml and stored at -20°C in small aliquots which were discarded after use.

8.5.3 Nucleon II cell lysis solution

10mM Tris-HCl, 320mM sucrose, 5mM $MgCl_2$, 1% Triton X-100

109.54g	sucrose
1.02g	$MgCl_2$
10mls	1M Tris (pH 8)
10mls	Triton X (add after autoclaving)

Made up to 1 litre in distilled water.

8.5.4 Nucleon II washing solution

400mM Tris-HCl, 60mM EDTA, 150mM NaCl, 1% SDS

2.19g	NaCl
100mls	1M Tris (pH 8)
30mls	0.5M EDTA
25mls	10% SDS (add after autoclaving)

Made up to 250mls in distilled water.

8.5.5 Digestion buffer for large scale gDNA extraction from brain

10mM Tris pH 8, 25mM EDTA pH 8, 100mM NaCl, 0.5%SDS

0.42g	NaCl
1ml	1M Tris (pH 8)
5ml	0.5M EDTA (pH 8)
5ml	10% SDS (add after autoclaving)

8.6 Isolation and manipulation of proteins

8.6.1 Pierce Protein Assay

Reagent A	sodium carbonate
	sodium bicarbonate
	BCA detection reagent
	sodium tartarate in 0.1M NaOH
Reagent B	4% copper sulphate.$5H_2O$

The standard protocol was employed, this measured protein in the range of 100 to 1200 µg/ml and used BCA(bicinchoninic acid) protein assay reagent

Reagent A	2ml
Reagent B	40µl
Protein Sample	100µl

The above were incubated at 37°C for 30 minutes and the optical density measured at 562nm.

8.6.2 Polyacrylamide gel compositions

8.6.2.1 Stock Solution: 40% acrylamide-bisacrylamide

380g	acrylamide (DNA sequencing grade)
20g	N,N`-methylenebisacrylamide
To 600ml	distilled water

Heat the solution to 37°C to dissolve the chemicals. Adjust the volume to 1litre with distilled water. Filter the solution through a nitrocellulose filter (Nalge, 0.45-micron pore size). Store in dark bottles at 4°C.

8.6.2.2 4% resolving gel

4.0ml	acrylamide-bisacrylamide (30:0.8)
3.75ml	resolving gel buffer stock
	(3M Tris-HCl (pH8.8))
0.3ml	10% SDS
0.7ml	1.5% ammonium persulphate
21.25ml	distilled water
15µl	TEMED (just before pouring gel)

8.6.2.3 10% resolving gel

10ml	acrylamide-bisacrylamide
3.75ml	resolving gel buffer stock
	(3M Tris-HCl (pH 8.8))
0.3ml	10% SDS
0.7ml	1.5% ammonium persulphate
12.75ml	distilled water
15µl	TEMED (just before pouring gel)

8.6.2.4 2.5% stacking gel

2.5ml	acrylamide-bisacrylamide (30:0.8)
5.0ml	stacking gel buffer stock
	(0.125M Tris-HCl (pH 6.8))
0.2ml	SDS
1.0ml	1.5% ammonium persulphate
11.3ml	water
0.015ml	TEMED

8.7 Bacteriological media

8.7.1 Luria-Bertani medium

10g	tryptone (Oxid)
5g	yeast extract (Oxid)
10g	sodium chloride

Make up to 1 litre in distilled water, adjust to pH 7 using ~0.2ml 5N sodium hydroxide. Sterilise by autoclaving for 20 minutes at 15 lb/in^2. Store at 4°C.

8.7.2 SOC medium

20g	tryptone (Oxid)
5g	yeast extract (Oxid)
0.5g	sodium chloride

Made up to 970ml in distilled water, add 10ml 250mM potassium chloride, adjust pH to 7.0 using 5N sodium hydroxide (~0.2ml). Autoclave for 20 minutes at 15lb/in^2. Cool to <60°C then add 20ml of filter sterilised 1M glucose solution. Store at 4°C.

9. Abbreviations

APES	3-aminopropyltriethoxy-silane
APS	Ammonium persulphate
ATP	Adenosine triphosphate
α-TTP	α-tocopherol transfer protein
AVED	Ataxia with vitamin E deficiency
BNF	Buffered neutral formaldehyde
bp	Base pairs
^{o}C	Degrees centigrade
cDNA	Complementary DNA
CDRM	Chronic degenerative radiculomyelopathy
cm	Centimetre
CNS	Central nervous system
Con A	Concanavalin A
cpm	Counts per minute
CV	Cresyl violet
DAB	3,4,3`,4`-tetraminobiphenyl hydrochloride
DM	Myotonic dystrophy
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRPLA	Dentatorubral-pallidoluysian atrophy
DTT	Dithiothrietol
DW	Distilled water
EACA	Epsilon aminocaproic acid
EDM	Equine degenerative myeloencephalopathy
EDTA	(Ethylenedinitrilo)tetraacetic acid
EM	Electron microscopy
EMG	Electromyograph
EMND	Equine motor neurone disease
EPO	Evening primrose oil
EST	Expressed sequence tag

FCE	Fibrocartilaginous embolism
FeLV	Feline leukaemia virus
FRAXA	Fragile X syndrome
FRAXE	A sub-group of fragile X syndrome
g	Gram
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
GSD	German shepherd dog
GUVS	Glasgow University Veterinary School
H&E	Haematoxylin and eosin
HD	Hip dysplasia
HPLC	High performance liquid chromatography
HRS	Haw river syndrome
i.d.	internal diameter
in. Hg	inches of mercury
kb	Kilobase
kDa	Kilo Dalton
K_2HPO_4	Potassium hydrogen phosphate
L	Litre
lb	Pound
LB	Luria Bertani
LCL	Lymphoblastoid cell line
LiCL	Lithium chloride
LVN	Lateral vestibular nucleus
M	Molar
Mb	Megabase
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
mM	Millimolar

MJD	Machado-Joseph disease
MW	Molecular weight
$NaAc^-$	Sodium acetate
NaCl	Sodium chloride
NaH_2PO_4	Sodium hydrogen phosphate
NaOH	Sodium hydroxide
ng	Nanogram
NGS	Normal goat serum
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin P
PNS	Peripheral nervous system
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNAse A	Ribonuclease A
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SBMA	Spinal and bulbar muscular atrophy (Kennedy`s Disease)
SCA1-7	Spinocerebellar ataxia 1-7
SCD	Subacute combined degeneration
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDW	Sterile distilled water
sec	Second
SpG	Spinal ganglion
sq. in.	Square inch
SSC	Standard sodium citrate
STR	Simple tandem repeat

TU	Transcription unit
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBP	Tocopherol-binding protein
TE	Tris EDTA
TEMED	N,N,N`,N`,-tetramethylethylenediamine
TLCK	Nα-p-tosyl-l-lysine chloro-methyl ketone
U	Unit
μCi	Microcurie
μg	Microgram
μl	Microlitre
μm	Micrometre
μm l^{-1}	Micromoles per litre
UTP	Uridine-5`-triphosphate
UV	Ultraviolet
VLDL	Very low density lipoprotein
w/v	Weight in volume
X-phos	5-bromo-4-chloro-3-indoyl-phosphate

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